LIMNOLOGY FOR THE ORNITHOLOGIST: FRESHWATER FATTY ACIDS PROVIDE A VITAL NUTRITIONAL SUBSIDY FOR RIPARIAN BIRDS

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LIMNOLOGY FOR THE ORNITHOLOGIST: FRESHWATER FATTY ACIDS PROVIDE A VITAL NUTRITIONAL SUBSIDY FOR RIPARIAN BIRDS

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ABSTRACT

Highly unsaturated omega-3 fatty acids (HUFAs), are physiologically vital nutrients that are scarce in nature. Several major groups of freshwater primary producers are rich in HUFAs while terrestrial primary producers contain little to no HUFAs, but do contain their molecular precursor. Unmet HUFA demand may lead to mismatches between animals and their resources, resulting in limitation. Studies suggest that HUFAs may to be essential nutrients for animals with access HUFA-rich resources while others appear to be efficient at synthesizing HUFAs from the molecular precursor. While HUFAs are considered essential for many freshwater animals, much less is known about the HUFA needs of wild animals in terrestrial ecosystems. Emerging freshwater insects may supply riparian predators with HUFAs, providing a crucial nutritional subsidy, but also making these consumers dependent upon freshwater HUFAs.

After synthesizing the literature on HUFAs in natural ecosystems, I explored how HUFA availability in nature shapes consumer HUFAs requirements and use of subsidies in two species of riparian birds, Tree Swallows (*Tachycineta bicolor*) and Eastern Phoebes (*Sayornis phoebe*). In laboratory studies, I found that chicks of both

species performed better on diets richer in HUFAs compared to diets richer in the HUFA precursor. In a field studies, I found that freshwater insects were significantly richer in HUFAs than were terrestrial insects. Freshwater insects provided Eastern Phoebes with HUFAs even when chicks consumed far more terrestrial than freshwater insects. In addition, while Eastern Phoebes consumed freshwater insects across the landscape, relative HUFA availability and other environmental factors had little effect on the degree to which they relied upon freshwater insect HUFA subsidies. Overall, this suggests that subsidies are likely to be important when there are major differences between the nutritional quality of local resources and of subsidies.

My work indicates that HUFAs can be critically important in natural terrestrial ecosystems. HUFAs are a crucial dimension of food quality for developmental performance in two riparian avian insectivores. In addition, even when freshwater subsidies to riparian areas are relatively small, they can have profound impacts in nature as sources of critical nutrients that are scarce in terrestrial ecosystems.

BIOGRAPHICAL SKETCH

Cornelia Wingfield Twining was born on May 22nd, 1989 in Boston, Massachusetts to parents Alex and Nell. During the great naming compromise of 1989, her parents decided to name her Cornelia for legal purposes, but call her Lily. After living with her architect parents in Boston and Old Lyme, Connecticut for the first two memory-poor years of life, Lily moved to Bronxville P.O.-Yonkers, New. In New York, she had a brief toddler modelling career for must-have preschooler brands like Hannah Anderson and DUPLOS. After this experience, her parents sent her off to reform school at the Dutch Reformed Church of Bronxville nursery school.

At age five, Lily joined the ranks of the lifers at Hackley School in Tarrytown, NY. At Hackley, she discovered a love of ecology and natural history in Mr. Retz's lower school science classes and later in Ms. Clingen's AP Biology class, which her headmaster once likened to "cramming information into students' brains like cramming food down a foie gras goose's throat." Lily was also fortunate enough to spend many hours exploring the outdoors all on her own around the coastline, fields, forests, and tidal mudflats near her grandparents' house in Old Lyme. As much as she loved the outdoors, Lily actually spent most of her free time during middle and upper school in a freezer year-round training as a competitive figure skater.

Lily next attended Yale College in New Haven, Connecticut where she graduated with a degree in Environmental Studies in 2011. Lily spent her freshman summer studying Mandarin Chinese at Harvard Beijing Academy on a Richard U. Light Fellowship. During her sophomore summer, she began working with Dr. David Post, discovering interests in freshwater ecology, food webs, and stable isotopes. David's lab was particularly attractive to her because they were doing research right down the road from her family's home in Old Lyme as well as at Linsley Pond, the site of many of G. Evelyn Hutchinson's foundational ecological studies. During Lily's

time in the Post Lab she was involved with several projects and became highly proficient at measuring *Bosmina* spp. butt spikes (a.k.a., mucros) and weighing out precise amounts of material into very tiny tins. Whilst in the Post Lab, she delved into paleoecology and completed an Environmental Studies senior thesis on the historical ecology of coastal Connecticut lakes, receiving a Gaylord Donnelly Prize in Environmental Studies. Lily had so much fun in the Post Lab that she decided to turn her senior thesis project into a Master's of Environmental Science through the Yale School of Forestry and Environmental Studies in 2012.

In Fall of 2012, Lily began her Ph. D. in Ecology and Evolutionary Biology (EEB) with Dr. Alex Flecker. After completing her A-exam in April 2014, she added Dr. Nelson Hairston Jr. as a co-advisor. At Cornell, Lily was the recipient of an NSF Graduate Research Fellowship, an NSF Doctoral Dissertation, a USDA Hatch Grant with Dr. Tom Brenna, and as well as funding from the Cornell Lab of Ornithology and Cornell College of Agriculture and Life Sciences. She received a Robert H. Whittaker Award for her talk at the EEB symposium and a Lamont C. Cole Award for one of her thesis papers. As a graduate student, Lily had the opportunity to mentor three senior thesis students: Keren Bitan, Sara Gonzalez, and Sarah Dzielski, as well as her longtime Environmental Science and Sustainability major mentee Diamond Oden.

During her first semester, Lily met her eventual husband, Jeremy Ryan Shipley, in the EEB Core Course in Fall 2012. They married on June 17th, 2017 with many Cornell EEB members in the wedding party and in attendance. Ryan introduced Lily to the advantages of working with charismatic, easy to observe fauna (i.e., birds), especially at a university with resources dedicated solely to ornithological research. She introduced Ryan to limnology in return, but was not as successful at converting him into a limnologist as he was in converting her into an ornithologist.

To Ryan, who actually made this Ph. D. possible

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Second, I want to thank my husband Ryan for providing both technical and emotional support throughout my Ph.D. Ryan has been the best collaborator that I could have ever asked for. He helped me collect birds and insects in the field, he taught me how to become a neonatal avian phlebotomist, he built me emergence traps, he raised many, many clutches of finicky Tree Swallow and Eastern Phoebe chicks in the lab for me, he taught me how to analyze data, and he conducted statistical analyses that were beyond me to answer questions with a large long-term dataset. Moreover, he listened to me endlessly bemoan about how I would never be able to accomplish everything that I wanted to do and would never be able to finish and find a job. Ryan believed in me and my ideas when nobody else did and helped make my crazy side project ideas into what became the core of my dissertation. He also renovated our house, teaching me new skills like dry walling, and took care of our pets during the day and all while working on his own Ph.D. and publishing prolifically.

I next want to thank my co-advisors, Alex Flecker and Nelson Hairston, and my committee members Tom Brenna, Cliff Kraft, and David Winkler. Alex and Nelson demonstrated how to have highly productive and mature scientific disagreements that were respectful and always pushed to consider more than one

writing style, school of thought, or conclusion, improving my work in the end. They were also both endlessly supportive of me delving into systems and methods that were brand-new to us all in order to answer exciting ecological questions. Tom welcomed me into his lab in spite of my complete lack of undergraduate training in biochemistry or physiology. Along with Pete Lawrence, Donghao Wang, and others in the Brenna Lab, he opened up a whole new toolbox and set of research questions for me. Cliff encouraged me at the Cornell Adirondack Field Station with Kurt Jirka and Dan Josephson during my first summer and continued to support me even when I decided to move on to working on birds around Ithaca instead. Cliff remains one of my favorite people at Cornell to talk about science generally with, not to mention U.S. politics and environmental policy. Wink was a later, post-A exam addition to my committee when it became clear that it would be helpful to have a committee member who knew something about ornithology. He welcomed me into his lab group, gamely setting me up with datasets and field systems in which to answer my questions. In addition, he was always a strong advocate for Ryan and me as a team and encouraged us to do the best science we could together rather than pushing Ryan, his own highly ambitious student, forward or forcing Ryan and me compete with each other.

My labmates, both formal and informal, throughout my time at Cornell were also key to my surviving graduate school. Chris Dalton gets special recognition for encouraging me to join David Post's lab as a lab alum when I was an undergraduate and by setting an example as not only as a model Post Lab undergrad, but also as a model Hairfleck graduate student. Sarah Collins continues to be a fantastically supportive lab big sister and has been an amazing example of how to balance work and life as a female academic. Keeley MacNeill and Erin Larson were with me going through some of the same grad school imposter syndrome and confidence struggles and the Flecker lab sisterhood that we have built has made us all more resilient.

Rachel Abbott Wilkins and Katie Sirianni, my Hairston lab sisters, were always some of the most engaged with my research and helpful in lab meetings even though I ended up working quite far from zooplankton. Lindsay Shaffner, my other Hairston lab sister, literally helped me many times when it came to setting up for fieldwork or working in the lab and also shared in the joys of home ownership and home renovation. Amelia Weiss and Tim Lambert joined the Flecker lab later on and always made me feel like a helpful and knowledgeable older labmate even when I insisted that I really didn't have any amazing advice to give. Beyond my immediate lab groups, Jenny Uehling and David Chang-VanOordt were the best pseudo-lab siblings that I could have hoped for and both became some of my best friends at Cornell.

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PREFACE

All animals are faced with numerous constraints in their quest to survive, grow, and reproduce in nature (Darwin 1859). Chief among those constraints are resource availability and composition (Lack 1954). Limitation can occur when there are nutritional mismatches between an animal's demands and its resource supply. Controls on growth and secondary production have long been an area of active research within ecology: early studies focused on food resource availability, demonstrating how energy was lost at each step through successive trophic levels from primary producers to animals (Elton 1933; Lindeman 1942). However, it soon became clear that animals could be limited by resources other than energy (e.g., Ohle 1956), often requiring specific nutrients (e.g., Schindler 1968; Moss 1969). Elemental nutrients like carbon, nitrogen, and phosphorus are the among best-studied nutrients from an ecological perspective in part because they can limit plants and animals alike. The field of ecological stoichiometry has shown how the relative availability of elemental nutrients to one another can dictate nutrient movement through individual animals and as well as whole ecosystems (Sterner and Elser 2002).

In addition to elemental nutrients, animals, unlike plants and other autotrophs, also require organic compounds including fats, carbohydrates, and proteins. Ratios of organic compounds can also limit secondary production rates and alter food web interactions (Simpson and Raubenheimer 1993). Thus, animals are faced with numerous axes of potential nutritional mismatch that they must overcome through altering foraging behavior in order to avoid suffering physiological consequences. Resource subsidies, or the movement of energy and nutrients from one ecosystem to another (Polis et al. 1997), provide some animals with an opportunity to escape conditions of both locally poor food availability or food quality. However, to date studies on subsidies have largely ignored resource quality, focusing on the size of subsidies from donor

ecosystems rather than the degree to which organisms in recipient ecosystems use and rely upon subsidies.

This dissertation illuminates how small fluxes of scarce, but physiologically important nutritional resources can subsidize recipient ecosystems, connecting food webs at the landscape scale. This dissertation explores these nutritional connections using both controlled laboratory experiments as well as field studies. It demonstrates how a group of organic nutrients, highly unsaturated omega-3 fatty acids (HUFAs), can serve as limiting nutrients for animals in natural ecosystems and how variation in HUFAs between ecosystems can dictate animal reliance on both local resources and subsidies.

In **Chapter One**, I review previously published ecological literature on HUFAs, and propose that the supply and demand of HUFAs, which are scarce in nature, may lead to resource limitation across natural ecosystems. I open by discussing the universal physiological importance of HUFA diversity for animals ranging from zooplankton to vertebrates. I then introduce readers to the stark dichotomy between HUFA availability among aquatic and terrestrial primary producers: data from previous studies show that aquatic primary producers are often rich in HUFAs while terrestrial primary producers contain little to no HUFAs, but do contain alpha linolenic acid (ALA), the molecular precursor of HUFAs. Next, I describe how other researchers have used fatty acids, including HUFAs, as qualitative and quantitative tracers for reconstructing food webs. Next, I discuss what is known about both direct and indirect ecological implications of HUFA limitation in natural systems including changes in behavior, species composition, secondary production rates, trophic transfer efficiency and cross-ecosystem subsidies. Finally, I finish my review by highlighting future research priorities for myself and others including a need for additional research on HUFA availability and demand in terrestrial systems, the importance

of HUFAs for higher order consumers, and the food web and ecosystem-scale effects of dietary mismatches for scarce nutritional resources like HUFAs.

Chapter Two presents the results of an experimental study on the importance of HUFAs for a higher order terrestrial consumer, the Tree Swallow (*Tachycineta bicolor*). Tree Swallows, which are often found around fresh water lakes and streams, forage on a mixture of freshwater and terrestrial insects that differ in HUFA content. To understand Tree Swallow requirements for HUFA-rich freshwater insects during development, I manipulated both the quantity and quality of food for chicks undergoing rapid growth in a full factorial design: diets varied in quantity and were either high in HUFAs and low in ALA, the shorter-chain omega-3 molecular precursor to HUFAs, or low in HUFAs and high in ALA. Overall, I found that dietary HUFA content was more important for Tree Swallow chick performance than food quantity. On high HUFA diets, chicks grew faster, were in better condition, had greater immunocompetence and lower basal metabolic rates compared to chicks on either low HUFA diet. I also found that increasing the quantity of the high HUFA diet resulted in improvements to all metrics of performance while increasing the quantity of the low HUFA diets only resulted in greater immunocompetence and lower metabolic rates. I analyzed chick fatty acid composition, finding that chicks preferentially retained HUFAs in brain and muscle when both diet and HUFAs were limited. I conclude that HUFAs are a crucial dimension of food quality for developmental performance in Tree Swallows and that HUFAs and freshwater insect availability may impact Tree Swallow breeding success in natural populations.

In **Chapter Three**, I use a combination of a laboratory study to demonstrate that HUFAs have important effects on developmental performance in another frequently riparian insectivorous bird, the Eastern Phoebe (*Sayornis phoebe*), and a field study to show that Eastern

Phoebes get their HUFAs from freshwater insects independently of the degree to which they rely upon freshwater insects for their total assimilated energy. I used bulk and compound-specific stable isotopes to determine where Eastern Phoebe chicks got their HUFAs and overall diet from in three representative stream and riparian ecosystems. I also raised chicks on high HUFA, low ALA or low HUFA, high ALA diets in the laboratory and monitored their growth and condition. Freshwater insects were not only significantly enriched in HUFAs compared with terrestrial insects, but they also provided Eastern Phoebes with HUFAs even when chicks consumed far more terrestrial than freshwater insects. HUFAs also increased Eastern Phoebe growth rate and condition during rapid development as they did in Tree Swallow chicks. Based on these findings, I conclude that even when freshwater subsidies to riparian areas are relatively small, they can have profound impacts in nature as sources of critical nutrients that are scarce in terrestrial ecosystems.

Chapter Four presents the results of a larger-scale field study on food quality, in terms of fatty acid composition, for and freshwater subsidies to Eastern Phoebes around eight streams along a forested to agricultural land use gradient. I found that even across sites, food quality differences were much greater between freshwater and terrestrial insects than they were among sites. Across the landscape, freshwater insects were significantly richer in HUFAs, largely driven by EPA content, while terrestrial insects, especially pollinators, were significantly richer in ALA, the HUFA precursor. Across sites, I found that Eastern Phoebe chicks relied on freshwater insects for 25-86% of their overall diet, but I did not find evidence that factors such as insect availability, insect fatty acid composition, or other local environmental factors, such as canopy cover, were associated with the strength of freshwater insect subsidies. Based on this result, I suggest that when there are major differences between the nutritional quality of local resources

and subsidies the degree of reliance on subsidized resources may be less important than high quality subsidy access itself.

Related Side Projects

To understand the real-world implications of Chapter Two in terms of the effects of freshwater insects on Tree Swallow breeding success in nature, with my co-authors and I analyzed a long-term (~30 year) dataset on Tree Swallow breeding metrics and aerial insect biomass in a related side project. In this study, we find that freshwater insect biomass is a strong, positive predictor of Tree Swallow chicks' ability to survive and leave their nests while terrestrial insects have no impacts on survival to fledge. Specifically, we find that freshwater insect biomass is most important during a chick's rapid growth stage, the same stage at which we found HUFAs to have strong effects on survival-relevant metrics of performance like body condition in our previous laboratory study. As HUFA are the key nutritional compound that differ between freshwater and terrestrial insects, we suggest that freshwater sources of HUFAs can have strong effects on terrestrial consumer performance in natural systems.

To better understand the physiological mechanisms that make HUFAs so valuable, I also examined Tree Swallow chick ability to convert ALA, the molecular precursor to HUFAs found in terrestrial as well as freshwater systems, into HUFAs in another related side project with co-authors (Twining et al. 2017). We fed wild chicks δ^{13}C-enriched ALA to see if they could convert it into δ^{13}C-enriched EPA and DHA at all, and if they were, how efficiently they were able to do so. This allowed us to determine if Tree Swallow chicks were directly limited by dietary HUFAs alone or if they were also indirectly limited by the costs of converting ALA into HUFAs. We found that Tree Swallow chicks were able to convert ALA into HUFAs, but that they were inefficient at doing so, suggesting that they suffer from direct as well as indirect

HUFA limitation. When we estimated how much HUFAs Tree Swallow chicks could derive from ALA in nature, we found they could get far more HUFAs from consuming freshwater insects than from converting ALA in terrestrial insects into HUFAs, providing a mechanism for our long-term study result. We argue that the availability of ALA in natural prey items combined with the conversion efficiency make HUFAs from freshwater systems ecologically essential nutrients.

Just as freshwater ecologists were among the first to focus on energy transfer (Lindeman 1942), ecological subsidies (Juday 1932), and food quality in terms of elemental nutrients (Sterner and Elser 2002), limnologists were also among the earliest to identify HUFAs as important resources in nature (Brett and Müller-Navarra 1997). While numerous studies have demonstrated that HUFAs are a critical metric of food quality for wild freshwater animals like zooplankton (e.g., Brett and Müller-Navarra 1997), research on HUFAs in natural terrestrial systems has been comparatively limited. My dissertation work suggests that food quality in terms of HUFAs can be critically important in natural terrestrial ecosystems. I have demonstrated that there is a major dichotomy in food quality between freshwater and terrestrial systems that exists at both the level of primary producers and consumers. I have also shown that terrestrial consumers with access to freshwater resources may be highly reliant upon freshwater subsidies for specific nutrients. In particular, I have shown that HUFAs are critical nutrients for two widespread species of insectivorous riparian birds. Together, my dissertation findings suggest that once again findings first demonstrated within limnology are highly germane to the ornithologist.

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CHAPTER 1

HIGHLY UNSATURATED FATTY ACIDS IN NATURE: WHAT WE KNOW AND WHAT DO WE STILL NEED TO LEARN?

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Abstract

The supply and demand of highly unsaturated omega-3 fatty acids (HUFA) in natural ecosystems may lead to resource limitation in a diverse array of animal taxa. Here, we review why food quality in terms of HUFAs is important, particularly for neural tissue, across a diversity of animal taxa ranging from invertebrate zooplankton to vertebrates (including humans). Our review is focused on HUFAs rather than other unsaturated fatty acids because these compounds are especially important biochemically, but scarce in nature. We discuss the dichotomy between HUFA availability between aquatic primary producers, which are often rich in these compounds, and terrestrial primary producers, which are contain little to none of them. We describe the use of fatty acids as qualitative and quantitative tracers for reconstructing animal diets in natural ecosystems. Next, we discuss both direct and indirect ecological implications of HUFA limitation at the individual, population, food web, and ecosystem scales, which include: changes in behavior, species composition, secondary production rates, trophic transfer efficiency and cross-ecosystem subsidies. We finish by highlighting future research priorities including a need for more research on HUFAs in terrestrial systems, more research their importance for higher order consumers, and more research on the food web and ecosystem-scale effects of HUFA limitation.

Introduction

The processes governing variation in food web productivity have long fascinated ecologists (Lindeman 1942). Top–down and bottom–up forces (e.g. predation and nutrients) together regulate ecosystem structure and function (Carpenter et al. 1985). Studies of bottom–up processes have focused attention upon ecosystem limitation by, and cycling of, inorganic nutrients such as nitrogen and phosphorus (Sterner and Elser 2002). However, unlike autotrophs, which use inorganic nutrients directly, animals require organic compounds in their diets to support energetic and physiological demands (Müller- Navarra 2008, Iverson 2009) and so may become limited not only by inorganic elemental nutrients but also by complex organic compounds, such as specific amino acids, sterols or fatty acids. Examining animal requirements for organic compounds is yielding important new insights into how consumers within and between trophic levels interact and how such interactions affect ecosystem function.

Thus far, ecologists have paid much less attention to the role of organic compounds in ecosystem processes than they have to the role of inorganic nutrients. The theory of ecological stoichiometry, or the relative availability of elemental nutrients within different food web components, has yielded important insights into the structure and functioning of ecosystems (Sterner and Elser 2002). Analyses of ratios of carbon to nitrogen, carbon to phosphorus, and nitrogen to phosphorus have become a standard ingredient in the toolkit of ecologists, from eco-physiologists to global biogeochemists. Yet, for animals, the form of nutrients may be as important as their relative abundances (Anderson and Pond 2000, Frost et al. 2005). For the most part, even in animal-centric studies, ecologists have concentrated on inorganic nutrient limitation, or have treated bulk carbon and nitrogen as surrogates for lipid and protein content, respectively (Karasov and Martinez del Rio 2007). Ecologists have largely left the study of

organic nutrients, such as essential fatty acids, amino acids and vitamins, to food scientists and animal nutritionists (Sargent et al. 2002, Simopoulos et al. 2002, 2004, Raes et al. 2004, Zdunczyk and Jankowski 2013), though notable exceptions exist, for example polyunsaturated fatty acids (PUFAs) and sterols within the zooplankton nutrition literature (Brett and Müller-Navarra 1997, Martin-Creuzberg et al. 2009). Most studies from the food science and animal nutrition literature have focused on a limited number of domesticated taxa (e.g. poultry or farm-raised fish and shellfish) under controlled laboratory conditions (Sargent et al. 1999, Raes et al. 2004, Zdunczyk and Jankowski 2013, Hixson et al. 2014). As a result, many basic ecological questions concerning organic nutrient limitation and demand remain unanswered for animals in natural terrestrial and aquatic ecosystems.

The quality of foods can be defined in several ways including the proportion of food composed of indigestible materials, (e.g. cellulose and lignin), the proportion of food containing of toxins, and the proportion composed of essential organic compounds (i.e. those that animals cannot synthesize de novo), which include a number of pigments and several fatty acids (Karasov and Martinez del Rio 2007). In this review, we focus on food quality limitation by the HUFAs eicosapentaenoic acid (20:5n-3, EPA), and docosahexaenoic acid (22:6n-3, DHA), which are biochemically important, but scarce in nature. We first explain why food quality is important and discuss differences in HUFA requirements for different animal taxa. Second, we review the literature on variation in the fatty acid composition of primary producers. Third, we discuss the potential for using fatty acids as qualitative and quantitative dietary tracers for reconstructing diets. We then highlight a number of ecological implications of HUFA limitation at the individual, population, food web, and ecosystem scales. We finish by emphasizing future research priorities including for: terrestrial systems, higher-order consumers, food web and

ecosystem-scale effects of HUFA limitation, animal HUFA requirements from a phylogenetic perspective, and research that applies laboratory findings on animal HUFA limitation to natural systems.

Potential for fatty acid limitation

Animals are capable of synthesizing some of the fats that they need by endogenous metabolism of organic carbon compounds, such as ingested sugars, and converting them to fatty acids for energy storage, synthesis of structural lipids in membranes, or signaling compounds (Desvillettes and Bec 2009, Taipale et al. 2011, Strandberg et al. 2014). Fats, such as triaclyglycerols, are the primary energy storage molecules for animals because compared with proteins or carbohydrates they are twice as energy-dense and can be stored with less water (Karasov and Martinez del Rio 2007). Many fats are also important components of cell membranes and play key roles in both hormonal and neural signaling pathways. Animals cannot synthesize de novo some of the most important fats that they need. Instead, they must obtain them or their precursors from their diets, creating the potential for limitation not by bulk organic carbon, but by particular organic compounds in the form of certain fatty acids (Brenna et al. 2014).

Polyunsaturated fatty acids (PUFAs), or fats with more than one double bond (Box 1), are physiologically important and so are likely candidates for food quality limitation in natural ecosystems because a diverse array of invertebrates and vertebrates, both aquatic and terrestrial, require them in their diets (Goulden and Place 1990, Ahlgren et al. 1997). A class of PUFAs with great potential for limitation in humans and a number of other animals, especially other vertebrates, are those that have their first of multiple double bonds on the third carbon atom from the methyl end of the fatty acid molecule (omega-3 PUFAs; Holman et al. 1963), such as alpha

linolenic acid (18:3n-3, ALA), and especially the highly unsaturated omega-3 fatty acids (HUFAs) EPA and DHA (Sargent et al. 1999, Brenna et al. 2009; Box 1.1). Omega-3 HUFAs serve a wide array of important physiological roles ranging from structural components and hormone regulation (Brett and Müller-Navarra 1997) to functional components of neural membranes, especially synapses, inflammation, and immune functions (Arts and Kohler 2009). Omega-6 fatty acids (i.e. those with their first double bond six carbon atoms from the methyl end of the carbon chain; Box 1.1), which are much more plentiful in the diets of most terrestrial animals, also serve a vast number of physiological functions in animals, such as substrates for eicosanoid signaling molecules, hormonal precursors for insects (Stanley-Samuelson et al. 1988), and hibernation cues in mammals (Ruf and Arnold 2008). Hundreds of experimental studies on the effects of dietary HUFAs in humans and other animals have documented detrimental effects of their deprivation including: decreased growth or weight loss, impaired sight and other sensory abilities, and growth of fatty liver tumors (Henderson and Tocher 1987, Brenna et al. 2014). In mammals, dietary levels of DHA are vital for proper visual and cognitive development perinatally (Brenna et al. 2009). In freshwater crustacean zooplankton, HUFAs are important for reproduction and egg development (Anderson and Pond 2000, Müller- Navarra et al. 2000, Brett et al. 2009, Chen et al. 2012). For example, Müller-Navarra et al. (2000) found that growth of the crustacean herbivore, *Daphnia magna*, as well as trophic transfer efficiency, and egg production decreased when their phytoplankton food sources shifted to a cyanobacteria- dominated species assemblage with little to no HUFAs.

In particular, all vertebrates and most invertebrate groups require the HUFA DHA for proper tissue function. Most require a dietary supply of DHA because its endogenous biosynthesis by desaturation from any omega-3 precursor (i.e. ALA, EPA or DPA [22:5n-3]; Fig.

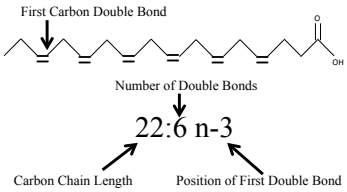
1.1b) is likely very limited, as it is in humans (Brenna et al. 2009). Endogenous DHA synthesis depends not only on the levels of DHA-precursors in the diet, but also on the mix of other dietary PUFAs, notably the omega-6 fatty acid linoleic acid (Brenna et al. 2009), which depresses endogenous DHA biosynthesis by competing for enzyme binding sites with the short-chain omega-3 PUFA, ALA (Brenna et al. 2009). When DHA synthesis is limited, animals require a direct dietary supply of DHA synthesized by other organisms in the food chain (Brenna et al. 2014). As with all fatty acids, DHA and its molecular precursors consumed in food are oxidized and incorporated into tissue, which can then be ingested and assimilated at the next trophic level (Brenna et al. 2014).

Animal species vary greatly in their HUFA demand depending on their ability to convert other PUFAs like ALA into DHA and EPA as well as their biochemical requirements. While nematodes (*Caenorhabditis elegans*) have even been found to convert omega-6 PUFAs into HUFAs (Spychalla et al. 1998, Kang et al. 2001), most animals appear to obtain EPA and DHA either directly from diet (Fig. 1.1a) or by elongating short chain omega-3 PUFAs (Fig. 1.1b). For example, vertebrates that have high levels of Δ6 and Δ5 desaturase enzymes are able to convert ALA into EPA and then DHA by removing hydrogen atoms (desaturation) and adding double carbon bonds (Bell and Tocher 2009; Fig. 1.1). Among animals that are unable to convert ALA into EPA and DHA, recent work suggests that DHA is preferentially retained and biomagnified

at higher trophic levels (Strandberg et al. 2015a).

Box 1.1 Fatty Acid Nomenclature

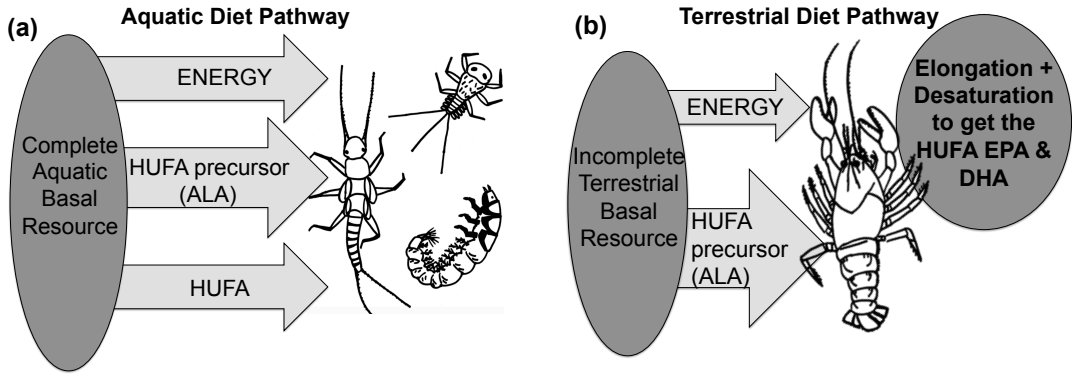
DHA: docosahexaenoic acid



Number of Double Bonds
Saturated Fatty Acids (SFAs): fatty acids without double bonds between carbon atoms
Monounsaturated Fatty Acids (MUFAs): fatty acids with one double bond between carbon atoms
Polyunsaturated Fatty Acids (PUFAs): fatty acids with multiple double bonds between carbon atoms
Highly Unsaturated Fatty Acids (HUFAs): polyunsaturated fatty acids with three or more double bonds

Position of First Double Bond
Omega-3 Fatty Acids: fatty acids with the first double bond on the third carbon atom from the methyl terminus
Omega-6 Fatty Acids: fatty acids with the first double bond on the sixth carbon atom from the methyl terminus

Figure 1.1 Direct and indirect pathways by which animals obtain HUFAs. **1.1a**: In the direct aquatic diet pathway, the consumer gets all of the HUFAs it needs directly from its aquatic food source. **1.1b**: In the indirect terrestrial diet pathway, the consumer gets only ALA (18:3n-3), the molecular precursor of EPA (20:5n-3) and DHA (22:6n-3), from its terrestrial food source and suffers a cost in terms of energy and trophic efficiency because it has to convert ALA into first EPA and then DHA through multiple steps of enzymatic processing.



Strong phylogenetic and trophic patterns in HUFA synthetic capacity occur among animals (Bell and Tocher 2009, Makhutova et al. 2011). For example, most marine fishes and many carnivorous fishes are unable to desaturate the short-chain omega-3 ALA (18:3n-3) into

either EPA (20:5n- 3) or DHA (22:6n-3), while herbivorous, detritivorous and omnivorous freshwater fishes retain some ability to desaturate ALA, albeit at an energetic cost (Tocher 2010, Vagner and Santigosa 2011, Castro et al. 2012). Freshwater grazing zooplankton are able to synthesize only a small portion of the HUFAs they need) and mostly accumulate them from diet (Goulden and Place 1990). For example, the ubiquitous freshwater grazer, *Daphnia spp*., has an ALA to EPA conversion efficiency of only 0.5% (von Elert 2002, Taipale et al. 2011). Terrestrial herbivores and omnivores, from chickens and anoles to rabbits and macaques, possess the Δ5 and Δ6 desaturase genomic architecture necessary to convert ALA to HUFAs more efficiently than aquatic organisms, such as fish (Castro et al. 2012), although exact rates of conversion efficiency across a diversity of taxa are scarce. In humans, average rates of ALA to DHA conversion are just under 5% (Brenna 2002). In contrast, strict terrestrial carnivores, such as cats, lack functional Δ6 desaturase and are unable to convert ALA to DHA and EPA (Pawlosky et al 1997). Because most work on fatty acid desaturation efficiency has been conducted on a limited number of lab-raised, domesticated, or hatchery-reared animals little is known directly about the desaturation capabilities and dietary HUFA requirements of wild animals, especially those in terrestrial systems (Hixson et al. 2015). In addition to selectively feeding on higher quality foods, at least some animals are capable of surviving by consuming poor quality food for brief periods if they have previously accumulated HUFAs under high food quality conditions either as polar lipids in phospholipid membranes, or as neutral lipids in fat reserves (Goulden and Place 1990, Hessen and Leu 2006, Brett et al. 2009, Gladyshev et al. 2011, Koussoroplis et al. 2013). Even small grazing invertebrates, such as daphniids, have the ability to preferentially accumulate HUFAs suggesting that even small animals with limited lipid storage capacity are under strong selection pressure to retain important compounds including

HUFAs from their diet (Goulden and Place 1990, Hessen and Leu 2006, Brett et al. 2009, Gladyshev et al. 2011, Taipale 2011, Koussoroplis et al. 2013). For example, Hessen and Leu (2006) found that *Daphnia* populations in lakes that differed in food quality were consistently enriched in EPA relative to the EPA content of seston. This mechanism allows animals to keep performing essential physiological processes using stored HUFAs even when they are scarce in diet. Experimental studies suggest that animals may be able to adjust their HUFA retention efficiency based on the quality of available foods in order to maintain a constant optimal level to carry out key physiological processes. Koussoroplis et al. (2013) found that *Daphnia magna* has the highest HUFA retention efficiency when fed HUFA- depleted diets. Research also suggests that animals preferentially use other fat stores as energy sources before resorting to metabolizing physiologically valuable HUFAs, thus retaining these important organic compounds to serve non-energetic functions (Brett et al. 2006, Gladyshev et al. 2011, Taipale et al. 2011).

Fatty acid sources

The potential for HUFA limitation in animals is based both on species' HUFA requirements as well as on the relative availability of HUFAs in their diets (Hixson et al. 2015). Animals must ultimately get HUFAs, or their molecular precursors short-chain omega-3 PUFAs, either from photosynthetic primary producers or from heterotrophic microbial gut symbionts (Russell and Nichols 1999, Sampedro et al. 2006, Bell and Tocher 2009). However, bacteria (Russell and Nichols 1999) as well as plants and other photoautotrophs (algae and cyanobacteria; Fig. 1.2–1.3) vary greatly in their fatty acid composition (Ahlgren et al. 1992, Taipale et al. 2013).

Few higher plants contain detectable amounts of HUFAs (Malainey et al. 1999, Mongrand et al. 2001, Simopoulos et al. 2002, 2004; Fig. 1.2a–c). Terrestrial vascular plants

tend to have a greater percentage of PUFAs as omega-6 fatty acids than non-vascular aquatic primary producers (i.e. algae and cyanobacteria) (Mongrand et al. 2001, Gladyshev et al. 2009, 2013; Fig. 1.2–1.3). In addition, the terrestrial plants for which there are data contain only short chain omega-3 fatty acids (Mongrand et al. 2001, Gladyshev et al. 2009, 2013), although Bryophytes, which are the closest terrestrial relatives of green algae, are a notable exception (Fig. 1.3). As a result, there are large differences in the fatty acid composition of terrestrial vascular plants and non-vascular aquatic primary producers in terms of both HUFAs and their short-chain omega-3 PUFA molecular precursors (Fig. 1.2a–c; Hixson et al. 2015).

Figure 1.2 Box plots of ALA, EPA, and DHA content in primary producers by ecosystem. **1.2**a: Percent ALA. **1.2b**: Percent EPA. **1.2c**: Percent DHA. Boxes represent the first and third quartiles, bars represent the highest and lowest datum within the 1.5 interquartile range, dots represent outliers, and n=x represents the sample size. Sources listed in Table S1.1.

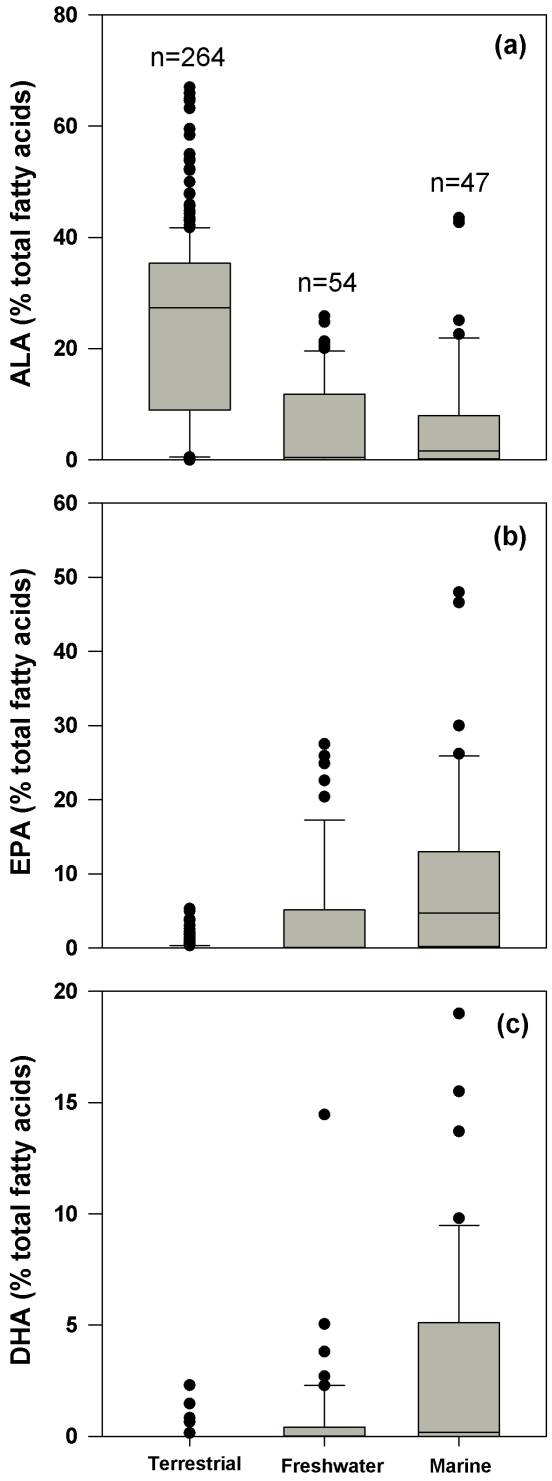
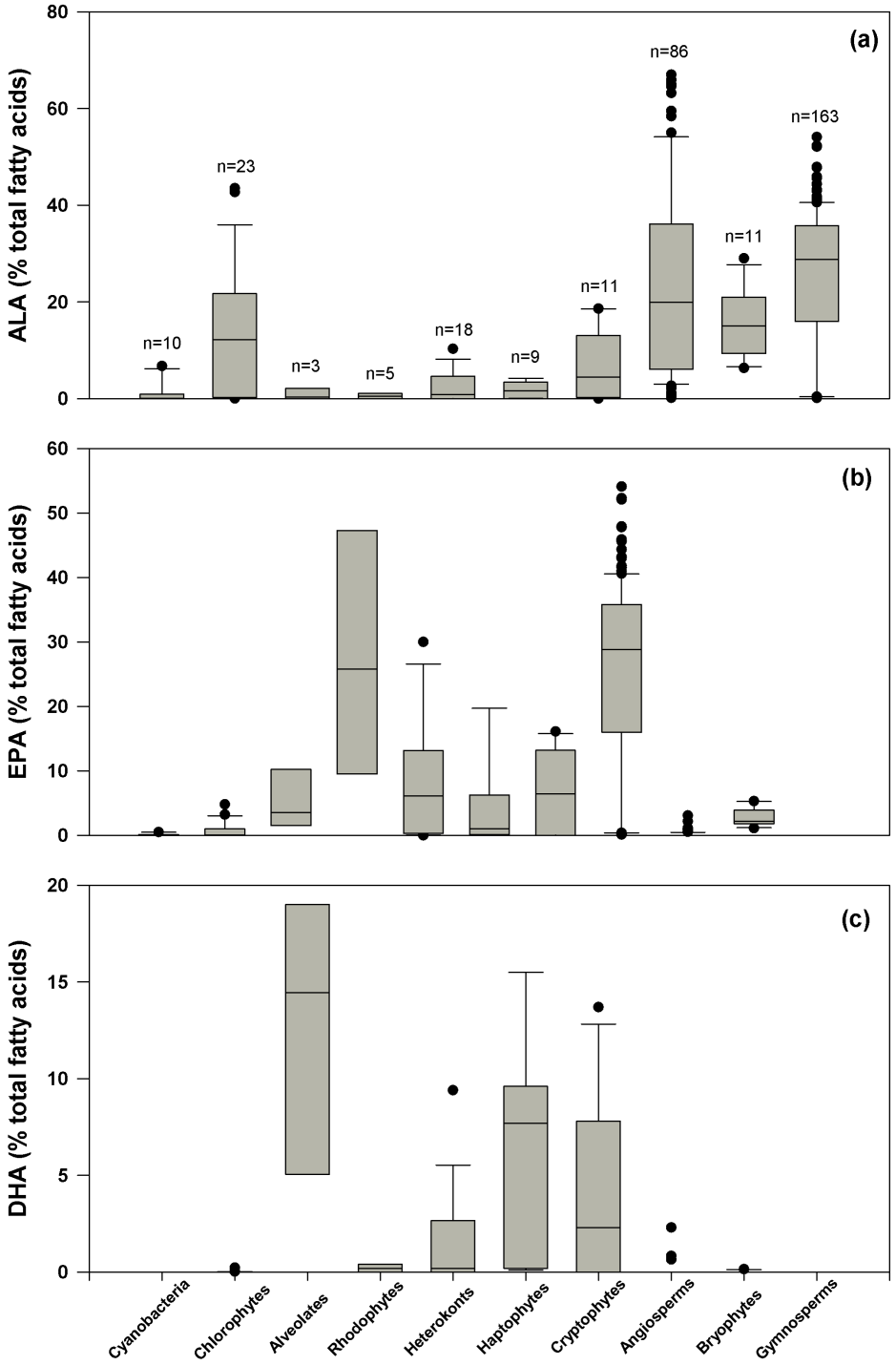


Figure 1.3 Box plots of ALA, EPA, and DHA content in primary producers by taxonomic group. **1.3a**: Percent ALA. **1.3b**: Percent. **1.3c**: Percent DHA. Boxes represent the first and third quartiles, bars represent the highest and lowest datum within the 1.5 interquartile range, dots represent outliers, and n=x represents the sample size. Sources listed in Table S1.1.



A key chemical feature of HUFAs is the susceptibility of their double bonds to chemical attack by both activated oxygen and a wide range of oxygenated radicals (Schepinov et al. 2014). The most unsaturated HUFA found in terrestrial animals, DHA, is particularly susceptible to oxidation due to its six double bonds. Whereas no purely terrestrial plant of which we are aware makes or accumulates DHA, this compound is abundant in non-vascular aquatic primary producers (Fig. 1.2a–c; Ahlgren et al. 1992, Taipale et al. 2013). In aquatic systems, oxygen transport is slowed by diffusion compared to transport by mass air movement and thus DHA in aquatic primary producers is easier to protect from oxidation.

However, aquatic primary producers themselves show enormous variation in fatty acid quality based on phylogeny as well as growth stage and environmental conditions (Guschina and Harwood 2009, Galloway and Winder 2015, Fig. 1.3). Algae grown at cooler temperatures have more total PUFAs as well as higher concentrations of HUFAs (Jiang and Gao 2004, Piepho et al. 2012) while high temperatures lead to a decrease in algal HUFA concentrations, which can have direct fitness effects on the zooplankton that graze on them (Sikora et al. 2014). Within both individual algal species and whole algal assemblages, high light conditions generally lower algal HUFAs, especially EPA, by causing oxidative damage (Napolitano 1994, Fabregas et al. 2004, Hill et al. 2011, Cashman et al. 2013). Some studies have found that growth limitation of algae by inorganic nutrients, such as phosphorus, increases HUFAs in some algal taxa (Arisz et al. 2000, Khozin-Goldberg and Cohen 2006, Hill et al. 2011). However, other studies have found that high levels of inorganic nutrients lead to decreased algal HUFA levels (Reitan et al. 1994, Cashman et al. 2013). Cashman et al. (2013) found that inorganic nutrients decreased DHA, EPA, and the ratio of omega-3 to omega-6 PUFAs even though they increased the short-chain omega-3 PUFA ALA. Abiotic factors including temperature, light and nutrients may also have

interactive effects on algal fatty acid composition (Hill et al. 2011, Piepho et al. 2012, Cashman et al. 2013). For example, Piepho et al. (2012) found that the influence of phosphorus availability varied in direction by genus and interacted with both light and temperature. In addition, research suggests that algae undergoing rapid growth under lab conditions have a lower proportion of HUFAs and a greater proportion of saturated and monounsaturated fatty acids than those in stationary growth phase (Brown et al. 1996). The contrasting responses of individual aquatic primary producer groups to abiotic forces make it challenging to predict the responses of entire communities to abiotic variation.

In contrast, there are strong phylogenetic patterns in fatty acid composition between different aquatic primary producer groups. For example, chlorophytes, the closest algal group to higher plants, contain high amounts of ALA, but little to no EPA or DHA, which one study suggested is due to their lack of necessary enzymes (Petkov and Garcia 2007), while the rhodophytes and heterokonts contain high amounts of EPA and the alveolates, haptophytes and cryptophytes contain high amounts of DHA (Fig. 1.3; Volkman et al. 1989, Ahlgren et al. 1992, Piepho et al. 2012, Galloway et al. 2012). In a recent meta-analysis, Galloway and Winder (2015) found that taxonomy accounted for three to four times more variation in phytoplankton fatty acid composition than nutrients, light, salinity or temperature, suggesting that taxonomic composition is the best predictor of phytoplankton HUFA availability. However, abiotic forces themselves exert strong selective pressures on aquatic primary producer community structure (Guschina and Harwood 2009). Thus, abiotic forces likely influence community- level aquatic primary producer fatty acid composition through their actions as environmental filters (Galloway et al. 2015).

Fatty acids as dietary tracers

The wide taxonomic and geographic variation in fatty acid composition of primary producers combined with the limited fatty acid desaturation and elongation capabilities of most animals make fatty acids a useful tool in place of or in combination with traditional diet reconstruction approaches, such as gut analyses or stable isotope analyses (Iverson 2009, Williams and Buck 2010). Controlled diet and complimentary gut content studies support the use of fatty acids as a tool for dietary reconstruction and suggest that the fatty acid signatures of consumers from small caddisflies to large mammals such as seals and seabirds generally resemble their food sources (Napolitano et al. 1996, Iverson 2004, Torres- Ruiz et al. 2010, Galloway et al. 2014). Omega-3 HUFAs as well as short-chain omega-3 and omega-6 PUFAs are especially useful as dietary tracers because: 1) they are minimally modified from ingestion to assimilation and tissue incorporation, and 2) they are generally conserved in their original forms to serve physiological functions instead of being broken down as energy sources or converted to other molecules. For example, Koussoroplis et al. (2008) used the ratio of DHA, which is much more abundant in aquatic systems, to linoleic acid (18:2n-6, LNA), which is well represented across both terrestrial and aquatic systems, to estimate the diets of carnivorous mammals that consumed different amounts aquatic and terrestrial prey items. They found that species, such as mink, that consumed mostly aquatic prey had much higher DHA in their adipose tissue than species, such as marten, that consumed terrestrial prey and had higher ALA levels (Koussoroplis et al. 2008).

In marine systems where fatty acid composition has been studied for decades (Jeffries 1970), researchers have even begun to take advantage of variation in fatty acid signatures for use as quantitative dietary tracers (Iverson et al. 2004, Iverson 2009, Williams and Buck 2010, Galloway et al. 2014). Researchers have used two: quantitative fatty acid signature analysis

(QFASA; Iverson et al. 2004, Iverson 2009) and fatty acid source tracking algorithm in R (FASTAR Galloway et al. 2015) to determine consumer diet composition down to the species level. For example, Iverson et al. (2004) used QFASA to correctly predict the preferred prey items of seals from a selection of 28 different prey species. QFASA uses a statistical weighting procedure to determine prey contribution to a predator's diet from a mixture of potential prey fatty acid signatures (Iverson et al. 2004) while FASTAR uses a modification of the Bayesian stable isotope mixing model MixSIR (Galloway et al. 2015). QFASA (Iverson et al. 2004) and FASTAR (Galloway et al. 2014) have the potential to be much more precise than stable isotope analyses. Even the best Bayesian stable isotope mixing models are limited to distinguishing between three to six food resources at best because they can only reliably discriminate between one more resource than the number of isotopes used (Parnell et al. 2010, Phillips et al. 2014). QFASA and FASTAR have the potential to incorporate information on more individual fatty acids than potential food sources in diet. In addition, while stable isotope signatures of resource species in the same environment at the same trophic level are often similar, quantitative fatty acid analyses can provide detailed species-specific information even within a given trophic level and ecosystem. For example, the 28 potential prey species of seals that Iverson et al. (2004) discriminated between were all marine fishes and shellfishes. Galloway et al. (2015) developed FASTAR to distinguish between phytoplankton taxa and Strandberg et al. (2015b) used FASTAR to determine phytoplankton community composition.

However, the presence of a given fatty acid in an animal's tissues may or may not reflect its presence in the animal's diet. To model an animal's diet comprehensively and accurately using fatty acids, researchers must know something about the species' lipid metabolism and fatty acid desaturation capacity in order to develop calibration coefficients for the consumer species in

question (Iverson et al. 2004, 2007, Iverson 2009, Williams and Buck 2010, Galloway et al. 2014). Thus, while they have high specificity, QFASA and FASTAR are more labor intensive than diet reconstruction techniques based on stable isotopes. This is because while animals obtain all elemental nutrients, such as carbon, nitrogen and phosphorus directly from their diets, they can obtain fatty acids either directly from diets or by modifying molecular precursors in their diets (Iverson 2004, 2009). As a consequence, researchers must use a secondary method of diet reconstruction, such as controlled feeding trials or gut analyses, for each consumer species of interest (Iverson et al. 2004, 2007, Torres-Ruiz et al. 2010, Galloway et al. 2014). While increasingly used, at present researchers have only developed calibration coefficients for a several marine seabirds and mammals (Iverson et al. 2007). Controlled feeding trials are not only time consuming, but are also limited to species that readily adapt to life under lab conditions while gut analyses only capture an animal's latest meal and require that study animals be sacrificed, something that may not be possible for legal or ethical reasons. Due to these challenges, QFASA and FASTAR have yet to become widely used tools for determining the diets of wild animals in natural systems.

An alternative approach to qualitative and quantitative fatty acid signature analyses involves using compound-specific stable isotope analyses to trace the pathways of selected fatty acids through entire food webs. Compound-specific stable isotope analyses allow researchers to trace the metabolic and food web pathways of individual fatty acids as opposed to merely following bulk carbon, and have been especially successful for tracing the pathways of PUFAs (Bec et al. 2011). They work best when the fatty acids of interest from different resources have distinct isotopic signatures (Bec et al. 2011). For example, Masclaux et al. (2013) revealed that several freshwater zooplankton taxa relied largely on inputs of terrestrial pollen grains that were

relatively enriched in $\delta^{13}C$ as their principal source of HUFAs, while relying on freshwater phytoplankton that was relatively depleted in $\delta^{13}C$ for their other fats. Thus far most compound-specific stable isotope work has focused on characterizing food web links in soil communities and in freshwater and marine systems (Reuss and Chamberlain 2010, Gladyshev et al. 2012). Researchers have also used compound-specific stable isotopes in mixing models to quantitatively determine where consumers derive their fats (Budge et al. 2008). Unfortunately, compound-specific stable isotope analyses are not only significantly more expensive than either bulk stable isotope analyses or fatty acid composition analyses, but are also not widely available at most institutions, and as a result ecologists have yet to adopt them widely.

Ecological effects of fatty acid limitation

In natural ecosystems, HUFA limitation can have both direct ecosystem effects, such as decreased growth and secondary production, as well as indirect effects, such as impaired neurological and hormonal function leading to behavioral changes with the potential for cascading effects on food web and ecosystem level processes. Decreased growth rate is the clearest direct effect of HUFA limitation for individual consumers. Laboratory studies across different animal taxa from invertebrate zooplankton to higher vertebrates including humans have documented decreased growth rates when consuming diets depleted in HUFAs (Sargent et al. 1999, Jakobsson et al. 2006, Zdunczyk and Jankowski 2013). However, studies conducted under highly controlled and simplified lab conditions have likely underestimated the full potential for HUFA limitation in natural ecosystems where caloric demands are greater due to increased activity levels, food availability is spatially patchy, and there are costs to foraging, such as evading predators and spending time away from young.

Fatty acid composition and availability also have the potential to play key roles in food web structure by limiting secondary production. A number of studies from the aquatic literature have documented the effects of HUFAs on the link between phytoplankton primary producers and zooplankton primary consumers (Brett and Müller-Navarra 1997, Müller-Navarra et al. 2000, 2004, Persson et al. 2007, Gladyshev et al. 2011). These studies suggest that zooplankton secondary production can be low even when elemental nutrients and food quantity are high if primary producers lack the HUFAs EPA and DHA (Müller-Navarra et al. 2000, Persson et al. 2007). Omega-3 HUFA availability may play an important role in food web structure by tightening the link between primary producers and secondary consumers (Müller-Navarra et al. 2000, 2004, Persson et al. 2007, Gladyshev et al. 2011; Fig. 1.1). Inefficient energy transfer may limit secondary production in taxa unable to adjust their digestive physiology and nutrient storage capacities to consume more total food in poor food quality conditions or may drive animals to consume more total food to obtain the same level of HUFAs via compensatory feeding (Raubenheimer and Simpson 1998; Fig. 1.1).

However, few studies have examined the effect that low HUFA availability at the base of the food web has on secondary or tertiary consumers. Emerging studies from the aquatic literature suggest that food quality in terms of HUFAs is likely highly important for the growth and survival of secondary and tertiary consumers in the wild (Volk and Kiffney 2012). For example, juvenile Coho salmon *Oncorhynchus kisutch* grow better and put on more fat when their diet is supplemented with marine salmonid carcasses rich in DHA (Heintz et al. 2004, 2010). HUFA availability for higher trophic level consumers may be driven by variation in the fatty acid composition of prey species: for example, cladocerans have higher percentages of EPA and while copepods have higher percentages of DHA (Gladyshev et al. 2015). A study on

sockeye salmon *Oncorhynchus nerka* suggested that populations of wild juvenile sockeye may be limited by the relatively low levels of DHA their prey, in particular *Daphnia* spp., which are the dominant food item in juvenile sockeye diets (Ballantyne et al. 2003). Few studies have examined the links between HUFA availability and secondary production in natural terrestrial systems. The few studies that have suggested potential for fatty acid limitation in wild terrestrial animals have focused on consumers that depend upon food resources from aquatic systems (Gladyshev et al. 2009, 2013). Gladyshev et al. (2009) estimated that although most terrestrial animals require some level of HUFAs directly from diet, some taxa, especially carnivores, may not be able to obtain sufficient amounts of HUFAs from consuming terrestrial-based foods alone. Gladyshev et al. (2009, 2013) suggest that emergent aquatic insects may transport HUFAs to some terrestrial consumers, such as riparian birds. In contrast, terrestrial matter appears to provide poor quality support for aquatic food webs due to its low lipid and HUFA content (Brett et al. 2009). However, in most cases, the ultimate sources of HUFAs for specific terrestrial consumers remain unresolved.

Dietary HUFA requirements may also make animals reliant upon nutrient subsidies from other ecosystems. Past work on nutrient subsidies has focused mainly on subsidy quantity (Polis et al. 2004) and paid little attention to subsidy quality. Studies that have looked at subsidy quality have characterized them only in terms of elemental nutrients and stoichiometry (but see Heintz et al. 2004, 2010, Gladyshev et al. 2009). Nevertheless, subsidy quality in terms of HUFAs may be of equal importance to quantity as well as quality in terms of elemental nutrients (Gladyshev et al. 2009, 2013). Nutrient subsidies from marine and freshwater systems to terrestrial systems are likely to be of higher quality than subsidies from terrestrial to aquatic systems because few terrestrial primary producers contain biologically significant amounts of HUFAs (Fig. 1.2a–c). In

addition, high HUFA animals actively dispersing, such as emergent insects, or being moved by other animals into terrestrial systems (e.g. bears dragging anadromous salmon carcasses out of streams) dominate subsidies from marine and freshwater to terrestrial systems, while low HUFA plant detritus passively falling into lakes and streams dominates subsidies from terrestrial to aquatic systems (Polis et al. 2004).

The dichotomy in the HUFA content of subsidies from terrestrial and aquatic systems is likely to drive HUFA movement through food webs (Fig. 1.2–1.3). For example, stream macroinvertebrates that feed on large fluxes of HUFA-deficient terrestrial detritus are likely to be limited by food quality (Torres-Ruiz et al. 2007). In contrast, riparian spiders and birds that rely on small, but high HUFA fluxes of emergent aquatic insects are more likely to be limited by food quantity (Gladyshev et al. 2013). However, the extent to which various animals rely on high quality subsidies of HUFAs from other ecosystems will likely vary based on their ability to desaturate and elongate ALA to EPA and DHA. For example, terrestrial consumers that have relatively efficient Δ6 desaturase enzymes are unlikely to require aquatic items in their diets, while terrestrial strict carnivores that lack functional Δ6 desaturase enzymes are more likely to rely on the occasional aquatic prey item.

Conclusions and future research priorities

In general, HUFA limitation likely plays an important role in structuring trophic interactions across taxa and trophic levels. Though they differ in their specific requirements and abilities to synthesize HUFAs from ALA, all animals require HUFAs. Primary producers at the base of food webs differ in both the quantity and quality of fatty acids. Omega-3 HUFA levels in primary producers may be far below what animals require in their diets, creating the potential for an

ecologically important mismatch between HUFA supply and demand. As a result, animals in natural ecosystems likely often suffer from HUFA limitation when both their synthesis capacity and dietary HUFA availability are low, such as terrestrial predators. Ecological consequences of HUFA limitation may include decreased growth rates, increased exposure to predation, and elevated stress responses, thus limiting secondary production, creating dependence upon HUFA subsidies at the landscape scale, or affecting ecosystem-level processes such as nutrient cycling and the magnitude of trophic cascades.

However, our knowledge about HUFA limitation and the role of essential fatty acids in ecosystems is far from complete. Several areas of critically-needed research stand out in particular: 1) omega-3 HUFA limitation is likely to be as important for terrestrial consumers as it is for aquatic consumers. Thus far the vast majority of HUFA limitation studies have been carried out in aquatic systems (Hixson et al. 2015). The importance of HUFAs, especially EPA, in zooplankton- phytoplankton interactions has been clearly established as has the potential for HUFA levels in primary producers to limit aquatic secondary production (Müller-Navarra and Lampert 1996, Müller-Navarra et al. 2000, 2004, Persson et al. 2007). How these findings translate to herbivore-primary producer interactions in natural terrestrial systems remains unclear (Hixson et al. 2015). Does HUFA availability in terrestrial plants limit secondary production herbivores? Indeed, the potential for HUFA limitation in terrestrial systems appears more likely than in aquatic systems because few terrestrial plants contain significant amounts of HUFAs (Gladyshev et al. 2009, 2013; Table 1). Do terrestrial plants from different biomes differ in the amount of HUFA, or HUFA precursors, that they contain (e.g. temperate and tropical rainforests with an abundance of *Bryophytes* in addition to other primary producers versus deserts with little primary production)? Consequently, are animals from different biomes adapted to local HUFA

availability? Laboratory studies on terrestrial animals suggest that they do require dietary HUFAs in their diets to the same degree as aquatic animals (Blomquist et al. 1991, Raes et al. 2004, Jakobsson et al. 2006, Zdunczyk and Jankowski 2013). However, if they are not limited by HUFAs to the same degree as aquatic consumers, are they able to convert long-chain omega-3 PUFAs into HUFAs or are they simply better at converting ALA into EPA and DHA (as can *C. elegans*; Spychalla et al. 2001, Kang et al. 2001)? If the latter is the case, do they do so using more efficient enzymes of their own or do they depend upon gut endosymbionts?

2) The majority of studies, both aquatic and terrestrial, have examined the potential for HUFA limitation at the herbivore-photoautotroph interface. Few studies in natural systems have examined the effects of HUFA limitation on carnivores or omnivores (but see Ballantyne et al. 2003). Do carnivores experience HUFA limitation to the same degree as herbivores? While animal tissue is in general of higher food quality than that of plants because animals themselves require HUFAs, mismatches may result between the HUFA needs of predators and the fatty acid composition of their prey, causing limitation in secondary and tertiary consumers. Studies linking predator fatty acid requirements, not just their fatty acid signatures, with the fatty acid composition of prey will be essential for understanding the effect of HUFA limitation on food webs.

3) Thus far, most research on fatty acid requirements and desaturation efficiency in animals has focused on a taxonomically limited group of organisms primarily composed of laboratory organisms without consistent regard to phylogeny or trophic position (but see Castro et al. 2012). The question still remains: does an animal's phylogenetic history or its diet determine its fatty acid physiology? At present both diet and phylogeny appear likely to be important in the evolution of animal fatty acid requirements and ability to obtain the HUFAs

needed from molecular precursors. However, few studies have examined fatty acid physiology within a phylogenetically-informed context (but see Makhutova et al. 2011). For example, while researchers know a great deal about the fatty acid requirements of a few species of farm animals, we know little about the needs of closely-related wild fowl or ungulates. In addition, lab studies on domesticated animals are likely biased by the fact that many of these taxa have been selected for fast growth on supplemented diets for hundreds to thousands of years. Selection for maximum growth very likely has led to the evolution of fatty acid requirements distinct from those of wild animals.

4) The food web and ecosystem implications of HUFA limitation remain largely unresolved in most systems (but see Müller-Navarra et al. 2000, 2004). Numerous studies on single consumers and their food sources have made clear that HUFAs can be limiting at the level of secondary production. However, few studies have examined either how HUFA limitation of consumers affects food web structure or biogeochemical processes. How does decreased food quality in terms of HUFAs at the base of the food chain, and its effect as a bottom–up force constraining production at intermediate trophic levels, compare with the top–down effect of predators (but see Litzow et al. 2006)? How does decreased food quality affect biogeochemical processes, such as carbon storage and nutrient recycling?

5) Finally, to understand the role of fatty acids in natural ecosystems, it will be necessary to test laboratory findings in nature. While the wealth of laboratory experiments in the fatty acid literature clearly suggests that HUFAs can limit secondary production, few studies have tested these findings in the field. How frequently do zooplankton in lakes experience EPA limitation compared with limitation by phosphorus or food abundance in natural lakes? Do salmonids in streams acquire the DHA that lab studies suggest they need for optimal growth? Do riparian

birds rely upon high quality subsidies of HUFA-rich emerging aquatic insects to feed their offspring? The answers to ecological questions such as these will only come through experiments in complex natural systems. Studies testing whether or not HUFA limitation occurs across natural ecosystems will enhance the relevance of laboratory studies for both ecologists and environmental managers tasked with increasing production of sport taxa or looking to increase the survival of threatened species.

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DATA DEPOSITION

Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.67dg6 (Twining et al. 2015).

CHAPTER 2

HIGHLY UNSATURATED OMEGA-3 FATTY ACIDS SUPPORT AERIAL INSECTIVORE PERFORMANCE MORE IMPORTANT THAN FOOD QUANTITY

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Abstract

Once-abundant aerial insectivores, such as the Tree Swallow (*Tachycineta bicolor*), have declined steadily in the past several decades, making it imperative to understand all aspects of their ecology. Aerial insectivores forage on a mixture of aquatic and terrestrial insects that differ in fatty acid composition, specifically highly unsaturated omega-3 polyunsaturated fatty acid (HUFA) content. Aquatic insects contain high levels of both HUFA and their precursor omega-3 PUFA, alpha-linolenic acid (ALA), while terrestrial insects contain much lower levels of both. We manipulated both the quantity and quality of food for Tree Swallow chicks in a full factorial design. Diets were either high HUFAs or low in HUFAs, but high in ALA, allowing us to separate the effects of direct HUFAs in diet from the ability of Tree Swallows to convert their precursor, ALA, into HUFAs. We found that fatty acid composition was more important for Tree Swallow chick performance than food quantity. On high HUFA diets, chicks grew faster, were in better condition, had greater immunocompetence and lower basal metabolic rates compared to chicks on both low HUFA diets. Increasing the quantity of high HUFA diets resulted in improvements to all metrics of performance while increasing the quantity of low HUFA diets only resulted in greater immunocompetence and lower metabolic rates. Chicks preferentially retained HUFA in brain and muscle when both food quantity and HUFA were limited. Our work suggests that fatty acid composition is an important dimension of aerial insectivore nutritional ecology and reinforces the importance of high quality aquatic habitat these declining birds.

Introduction

Aerial insectivores, a paraphyletic group that includes the swallows, swifts, nightjars, and at least five different families of flycatchers, were once abundant throughout both temperate and tropical regions. However, in the last half century, a number of North American aerial insectivores across a diversity of families and species, ranging from Common Nighthawks (*Chordeiles minor*) and Chimney Swifts (*Chaetura pelagica*) to Olive-sided Flycatchers (*Contopus cooperi*) and Tree Swallows (*Tachycineta bicolor*), have undergone major declines (Nebel et al. 2010, Sauer et al. 2014). For example, Tree Swallows, one of the best-studied model aerial insectivore taxa in North America, have declined by 36% over the past 2-3 decades (Sauer et al. 2014). Experts have proposed several hypotheses including: 1) declines in aerial insects (Paquette et al. 2013), 2) habitat loss and degradation (Paquette et al. 2013, Fraser et al. 2012, Robillard et al. 2013), 3) environmental contaminants (Alberts et al. 2013, Rowse et al. 2014), and 4) climate change and phenological mismatch (Lyon et al. 2008, Dunn et al. 2011). Evidence exists to support all of these hypotheses yet, at present, the exact causes of aerial insectivore declines remain unresolved, pointing to the need for a more thorough understanding of all aspects of aerial insectivore ecology.

Past studies have documented the importance of food resources for aerial insectivores and numerous studies have suggested that aerial insectivore declines are linked to decreasing overall insect abundance (e.g. Nebel et al. 2010). Research on Tree Swallows shows that food availability is linked with chick growth rates and fledging success as well as egg size and composition (Ardia et al. 2006, Winkler et al. 2013). Winkler et al. (2013) found that environmental temperature had a strong effect on patterns of Tree Swallow chick mortality, most likely through its effect on insect activity levels. However, the sheer quantity of food resources

may not be the only important factor. Food quality, and the potential for mismatch between insect composition and the nutritional needs of aerial insectivores may also be important drivers of reproductive output and overall fitness for these birds (e.g., Bidwell et al. 2005).

Food quality can be defined in many ways, including caloric density, nutrient composition, and digestibility (Karasov and del Rio 2007). Here, we focus on differences in composition of macronutrients. Aerial insectivores, like all animals, require organic compounds (e.g., vitamins, amino acids, and fatty acids) in addition to elemental nutrients (e.g., nitrogen, phosphorus, and calcium) to grow, develop, and complete their life cycles. Omega-3 highly unsaturated fatty acids (HUFA), in particular the fatty acids docosahexaenoic acid (22:6n-3, DHA) and eicosapentaenoic acid (20:5n-3, EPA), are especially important organic compounds for most animals, affecting a range of important physiological processes from immune function to vision and brain development (Twining et al. 2016). Birds and all other vertebrates must either consume EPA and DHA directly from diet or indirectly by consuming their molecular precursor, the short chain omega-3 PUFA, alpha linolenic acid (18:3n-3, ALA), and then converting ALA into EPA and DHA. The capability of any particular animal species to convert ALA to the bioactive EPA and DHA depends on whether its diet contains EPA and DHA (Twining et al. 2016). Mammalian herbivores typically synthesize all EPA and DHA endogenously from ALA, while carnivores such as cats must obtain all of their DHA from diet (Rivers et al. 1975). The ability of wild birds to synthesize DHA is not well characterized, but DHA concentrations are inversely related to mass (Hulbert et al. 2002). For example, DHA constitutes 12% of fatty acids in the muscles of House Sparrow (*Passer domesticus*; Hulbert et al. 2002), which are similar in size to Tree Swallows, but can reach over 20% in Ruby-throated Hummingbird muscle (*Archilochus colubris*; Infante et al. 2001).

In the wild, aerial insectivores consume a combination of terrestrial and aquatic insects (McCarty and Winkler 1999), which differ in their fatty acid composition (Hixson et al. 2015). Aquatic insects contain much higher levels of HUFAs than do terrestrial insects, a difference driven by differences in the fatty acid composition of aquatic and terrestrial primary producers (Gladyshev et al. 2009, Hixson et al. 2015). Aquatic primary producers, such as diatoms and dinoflagellates are rich in EPA and DHA (Galloway and Winder 2015), which can be incorporated into aquatic insect tissue (Torres-Ruiz et al. 2007). In contrast, vascular terrestrial plants contain little to no HUFAs, but do contain their molecular precursor ALA (Twining et al. 2016), which can be either incorporated into tissue or converted to HUFAs to a minor degree by terrestrial insects (Hixson et al. 2015). As a consequence, from the perspective of HUFA content, aquatic insects may constitute a higher quality food for aerial insectivores than do terrestrial insects.

However, because both aquatic and terrestrial insects contain ALA, the relative value of aquatic insects depends on the capacity of aerial insectivores to convert ALA into HUFAs (19). The ability to elongate ALA into HUFA varies greatly across taxa: strict carnivores, such as cats (Pawlosky et al. 1997, Castro et al. 2012), and animals from environments rich in HUFA, including most marine fish (Sargent et al. 1999), have lost the ability to elongate ALA into HUFA and must obtain them directly from diet. In contrast, terrestrial herbivores appear to be relatively efficient at converting ALA to HUFA (Jakobsson et al. 2006). The capacity of aerial insectivores to convert ALA to HUFA remains untested, but as predators living around riparian areas with emergent aquatic insects rich in HUFA they appear likely to be limited by HUFA content in diet.

The majority of past studies on avian fatty acid requirements have focused on

domesticated herbivorous taxa, especially chickens (e.g., Lin et al. 1991, Newman et al. 2002). These studies found domestic hens to be relatively efficient at elongating ALA EPA and DHA (e.g., Lin et al. 1991, Newman et al. 2002). Far fewer studies have experimentally manipulated dietary fatty acid composition for wild birds (but see McWilliams et al. 2002, Egeler et al. 2003, Pierce et al. 2005, McCue et al. 2009). These studies have found that both dietary composition and elongation capacity of individual species affect avian fatty acid composition (McCue et al. 2009). However, with the exception of work by Pierce et al. (Pierce et al. 2005) on Red-Eyed Vireos (*Vireo olivaceus*), these studies have either looked at seed and fruit-eating passerines or fish-eating seabirds. To our knowledge, no studies have explicitly examined the omega-3 fatty acid requirements of any aerial insectivores. Therefore, we sought to understand the importance of food fatty acid composition for aerial insectivores by varying both food quality and quantity in a balanced factorial experimental design.

In nature, the effects of food quality and quantity may be confounded because parents may provide chicks with an increased quantity of food to make up for low quality food. To address this, we experimentally manipulated both the quantity and fatty acid composition of food for wild-hatched nestling Tree Swallow chicks. Chicks were fed one of four diets: 1) high HUFA, a high quantity diet containing EPA and DHA (Hh), 2) a low HUFA, high quantity diet containing high ALA and low omega-3 HUFA (Lh), 3) a high HUFA, low quantity diet (Hl), and 4) a low HUFA, low quantity diet (Ll). We assessed size-specific growth rates, body condition, immunocompetence, and basal metabolic rates (BMR) as metrics of performance. We also determined the fatty acid composition of brain and breast muscle tissue from a subset of chicks from each treatment group.

Methods

We collected 44 wild Tree Swallow chicks from nest boxes around Ithaca, New York from 29 May 2015 to 7 June 2015. To prevent parental abandonment of chicks, we removed all chicks from each nest box. All animal work was approved under Cornell Institutional Animal Care and Use Committee protocol 2001-0051, New York State Department of Environmental Conservation scientific collection permit 1477, and United States Fish and Wildlife Service migratory bird scientific collection permit 757670.

Upon return to the lab, we weighed and sorted chicks into groups of 3-4 birds to receive one of 4 feeding treatments: 1) a high HUFA, high quantity diet containing EPA and (Hh), 2) a low HUFA, high quantity diet containing ALA, but no HUFA (Lh), 3) a high LCPUFA, low quantity diet (Hl), and 4) a low HUFA, low quantity diet (Ll). The two diets were not significantly different in calories, moisture, crude protein, or crude fat (Supplementary Table 2.1) and differed only in fatty acid composition. All diets were based upon standard commercial Mazuri ® nestling feed: Mazuri.com/mazurihandfeedingdiets-1.aspx. Standard nestling diets contained soybean oil as their principal fat source. Our high HUFA diets included a substitution of stabilized menhaden oil for soybean oil in a ratio of 7:3 while low HUFA diets included a substitution of flax oil for soybean oil in a ratio of 1:3. The resulting high HUFA diets contained approximately 1.82% ALA, 3.74% EPA, and 3.44% DHA while low HUFA diets contained approximately 6.25% ALA, 1.47% EPA, and 1.42% DHA (Table S1). Low quantity chicks were fed 4.5% of body mass per feeding session (the point at which begging still occurred at the end of the session) and high quantity chicks were fed 6% of body mass per session (produced chick satiation at the end of the feeding session) of body mass per feeding. The two diets were not significantly different in calories, crude protein, or crude fat. Caloric content was measured

through bomb calorimetry at the Cornell University Human Nutritional Chemistry Service Laboratory. Additional feed composition analyses were conducted by the Dairy One Forage Lab (Dairy One Cooperative, Ithaca, NY USA).

We grouped chicks of similar initial sizes together in nest groups so as to avoid under or over-feeding individual chicks and randomly split up chicks from the same clutch to avoid genetic effects. There were multiple replicates of each food quality and quantity treatment that covered the full range of initial chick masses.

We labeled each chick with nail polish on its head to distinguish between individuals within nest groups. Nests consisted of folded paper towel layers in plastic bowls. Bedding was changed approximately hourly or when soiled with feces from 7am-7pm. Two nests bowls were placed in a plastic box covered with a towel and placed the box over a heating mat equipped with a thermostat placed within the nest. Nest temperatures were kept at ~30ºC throughout the experiment and chicks experienced 12 hours of light and 12 hours of dark. We cleaned chicks with baby wipes to remove food and fecal residue twice per day.

Chicks were fed for approximately 12 hours a day when at least half of chicks on the high quantity diets were begging. All chicks were fed via 1mL sterile syringes that were washed between feedings and replaced daily. Feeds were made up daily by blending together a 2:1 ratio of feed to water and then refrigerating the mixture until needed. A subset of feed was re-blended and warmed to ~40ºC in a water bath every 2 hours.

Each chick was weighed four times daily with an Ohaus Scout Pro balance and the average of that mass was used for calculations. We also measured the head-bill and tarsus length of each chick to the nearest 0.01 mm a minimum of 2 times over the course of the experiment with Mitutoyo Digimatic 500 calipers. Growth rates were calculated as: [ln(mass or length on

day x) – ln(mass or length on day 0)] / (day x – day 0) and body condition was calculated as both the ratio of mass to head-bill length and the ratio of mass to tarsus length.

To measure immunocompetence, we used the simplified protocol described by Smits et al. (Smits et al. 1999). Chicks were injected with a treatment dosage of 100 µg of PHA-P dissolved in 20µl of PBS (Sigma–Aldrich, St Louis, MO, U.S.A.; see Vinkler et al. 2010). We measured the initial thickness of the patagium and injected PHA solution into the middle of the patagium, making a mark of the injection site and then measured the magnitude of the swelling reaction again after 6 ± 0.5 hrs (for the usage of a 6 h period see e.g. Møller et al. 2003; recently Bonato et al. (2009) have shown that there is no statistical difference in the PHA response between 6 h and 24 h after injection). Tissue thickness at the injection site was measured three times with (accuracy 0.01 mm) a digital micrometer (Mitutoyo) by applying pressure to the point where the skin is lightly moved from pressure of the micrometer, and using the average of these three measures for further analysis. The PHA-induced swelling response index was calculated as the average tissue thickness 6h after the treatment divided by the average thickness before the PHA injection.

To determine basal metabolic rate (BMR), we used an open-flow pull-mode FoxBox respirometry setup coupled with a climate controlled chamber at a flow rate of ~490 mL/min following the methods of Lighton (2008). We acclimated chicks to a T_a of ~33°C for 2 hours and took respirometry measurements in a multiplexed setup for 90 minutes where respired gas was sampled from each individual's chambers for a period of 10 minutes. To account for sensor drift, we took baseline measurements lasting 10 minutes every 30 minutes and after the completion of the trial. A 3 m copper coil constructed of 6.35 I.D. mm tubing was placed in-line upstream inside the respiration chamber to equilibrate the temperature between the chamber and the

incurrent airstream.

Respired gas was analyzed using a Sable Systems FoxBox field oxygen analyzer in a pull setup following the recommendations in Lighton (2008) where samples were scrubbed of water vapor before CO_2 measurements and CO_2 and water vapor before O_2 measurements using a combination of Drierite (W. A. Hammond DRIERITE Co. LTD Xenia, OH), Soda Lime, and Ascarite (Sigma-Aldrich P/N 223913). All Drierite was exposed to ambient CO_2 conditions for a minimum of 2 minutes to equilibrate with ambient atmospheric conditions (White et al. 2006). Gas samples were corrected for dilution effects through the measurements of water vapor pressure and atmospheric pressure throughout the experiment (Lighton and Halsey 2011). At the beginning and end of each experiment, flow rate calibration was performed by measuring the time to displace an inverted graduated cylinder of water of a known volume with excurrent air (Lighton and Halsey 2011). All airstream connection tubing was 6.35mm I.D. Bev-a-line IV.

Uncompensated flow rate (FR_u) was corrected into STP corrected flow rate (FR_i) measured from the FoxBox using the following formula: (1) $FR_i = FR_u * (kPa – WVP)/ kPa$, where kPa is the atmospheric pressure and WVP is the water vapor pressure (both in kilopascals). The rate of oxygen consumption was calculated using equation 11.1 in Lighton (2008) from the incurrent (subscript i) and the excurrent (subscript e) airstream measurements: (2) $VO_2 = FR_i (F_iO_2 - F_eO_2) / (1 – F_iO_2)$, where FRi is the rate for unscrubbed incurrent air, FiO_2 is the measurement from the baseline incurrent sample, FeO_2 is the rate from the sample of exhaled carbon dioxide free of water vapor (Drierite) and carbon dioxide (Soda Lime and Ascarite). Rate of carbon dioxide production was calculated using Equation 11.6 in Lighton (2008) from the incurrent (subscript i) and the excurrent (subscript e) airstream measurements: (3) $VCO_2 = [FR_e (F_eCO_2 – F_iCO_2) - F_eCO_2(VO_2)] / (1 – F_eCO_2)$. With these measures, the rate of carbon dioxide

production and oxygen consumption during our respirometry trials were then converted into a mass specific rate of energy use per unit of time (hour) using the following formula: (4) MR(mL O_2 • hr• g) = $VO_{2(mL)}$*(60) / (individual mass), where the metabolic rate (MR) is the volume of oxygen consumed multiplied by the number of minutes in an hour divided by the mass of the organism.

We determined the whole tissue fatty acid (FA) composition of brain and pectoral muscle for a sub-set of chicks from each treatment (n=4). After euthanasia, we dissected and weighed out brain and pectoral muscle samples from four chicks per treatment. FA methyl esters (FAMEs) were extracted from whole tissues using a modified one-step method (Garces and Mancha 1993, Zhou et al. 2008) and quantified using a BPX-70 (SGE inc.) column and a HP5890 series II GC-FID. Chromatogram data was processed using PeakSimple. Response factors were calculated using the reference standard 462a (Nucheck prep). FAMEs were identified using a Varian Saturn 2000 ion trap with a Varian Star 3400 gas chromatography mass spectrometer run in chemical ionization mass spectrometry mode using Acetonitrile as reagent gas. FA composition data is expressed as percent of total FA. We also calculated total omega-3 PUFAs and total omega-6 PUFAs.

We analyzed mass, size-specific growth rates for mass, tarsus length, size-specific growth rates for tarsus length, head-bill length, size-specific growth rates for head-bill length, the ratio of mass to tarsus length, the ratio of mass to head-bill length, and PHA ratio through ANOVA, using treatment group (the interaction of HUFA content and food quantity: Hh, Hl, Lh, and Ll), nest, and individual as predictor variables. For all performance metrics except PHA ratio, which was only measured at the end of the experiment, we also ran ANCOVA with experiment date as a covariate. We used post-Hoc Tukey tests to interpret the direction and significance of

differences between treatment groups for variables that were significant as main-effects and assessed relative support between models using AIC. To detect differences in our smaller datasets on basal metabolic rates and brain and muscle FA composition, we used non-parametric Kruskal-Wallis tests and performed Dunn tests to perform pairwise comparisons between treatment groups (Hh, Hl, Lh, and Ll). We also compared differences between brain and muscle fatty acid composition using Welch's two-sample t-tests. All statistical analyses were performed in R (3.2.2).

Results

Chicks on high HUFA diets grew significantly more rapidly than those on low HUFA diets regardless of food quantity (ANOVA: treatment $F_{3,128} = 59.889$, $p < 0.0001$; Figures 2.1a-b, 2.2). Diet quality was more important than quantity: even Hl chicks grew significantly faster than did Lh chicks (Table 2.1; Figures 2.1b, 2.2). Among the high LCPUFA groups, Hh chicks grew significantly faster than did Hl chicks (Table 2.1; Figures 2.1b, 2.2). Among the low HUFA groups, there were no significant differences between Ll or Lh chicks (Table 2.1). There were no significant treatment differences in head-bill or tarsus growth rates between treatments (ANOVA for head-bill: treatment $F_{3,90} = 0.099$, $p = 0.96$; ANOVA for tarsus: treatment $F_{3,90} = 2.091$, $p = 0.107$; Figure 2.1c). Thus, the differences observed were in growth rates for mass, not structural size.

Table 2.1 Size-specific growth rates analysis of variance and Tukey post-hoc tests

		Mass growth rate		
Variable	Degrees of Freedom	F-value	P-value	Tukey Post-Hoc Test for Treatment Group
Date	3	2.565	< 0.1	Ll < Hh (< 0.001)
Treatment Group	3	59.889	< 0.0001	Ll < Hl (< 0.001) Ll =Lh
Nest	8	10.917	< 0.0001	Lh < Hh (< 0.001)
Individual	32	6.954	< 0.0001	Lh < Hl (< 0.001)
Residuals	128			Hl < Hh (< 0.001)
		Tarsus growth rate		
Variable	Degrees of Freedom	F-value	P-value	Tukey Post-Hoc Test for Treatment Group
Treatment Group	3	2.091	NS	NS
Residuals	90			
		Headbill growth rate		
Variable	Degrees of Freedom	F-value	P-value	Tukey Post-Hoc Test for Treatment Group
Treatment Group	3	0.099	NS	NS
Residuals	90			

Chicks on high HUFA diets were also in significantly better condition (reflected by the ratio of mass to head-bill length and mass to tarsus length) than those on low HUFA diets regardless of quantity (ANOVA for mass to tarsus: treatment $F_{3,115} = 225.673$, $p < 0.0001$; ANOVA for mass to head-bill: treatment $F_{3,115} = 276.462$, $p < 0.0001$; Figure 2.1d). Chicks on Hh were in significantly better condition than were Hl chicks (Table 2.2), and Hl chicks were in significantly better condition than were Lh chicks (Table 2.2; Figure 2.1d). Among the low HUFA groups, chicks on Lh were in significantly better condition than were Ll chicks (Table 2.2).

Table 2.2 Body condition analysis of variance and Tukey post-hoc tests

Mass to tarsus ratio

Variable	Degrees of Freedom	F-value	P-value	Tukey Post-Hoc Test for Treatment Group
Date	4	11.632	< 0.0001	Ll < Hh (< 0.001)
Treatment Group	3	225.673	< 0.0001	Ll < Hl (< 0.001)
Nest	8	101.571	< 0.0001	Ll < Lh (< 0.001)
Individual	7	3.369	< 0.01	Lh < Hh (< 0.001)
Residuals	115			Lh < Hl (< 0.001)
				Hl < Hh (< 0.001)

Mass to headbill ratio

Variable	Degrees of Freedom	F-value	P-value	Tukey Post-Hoc Test for Treatment Group
Date	4	14.397	< 0.0001	Ll < Hh (< 0.001)
Treatment Group	3	276.462	< 0.0001	Ll < Hl (< 0.001)
Nest	8	131.713	< 0.0001	Ll < Lh (< 0.001)
Individual	7	2.755	< 0.05	Lh < Hh (< 0.001)
Residuals	115			Lh < Hl (< 0.001)
				Hl < Hh (< 0.001)

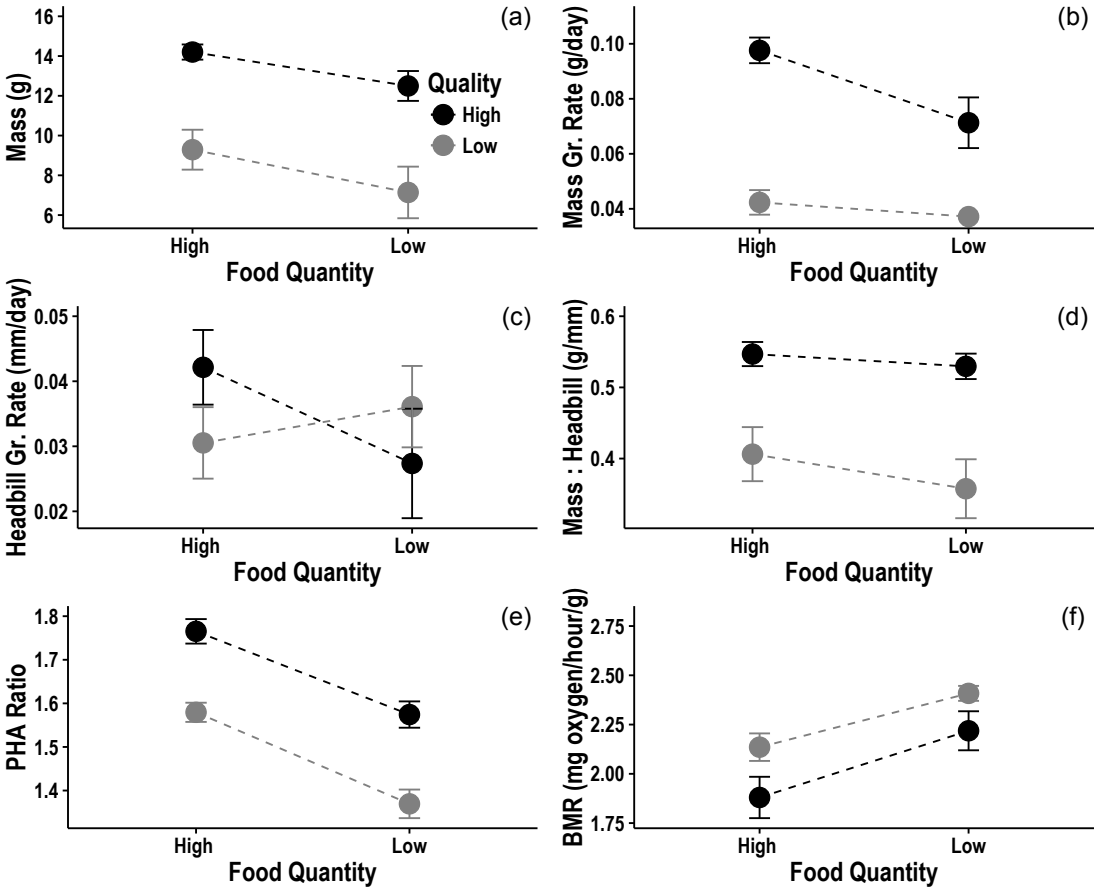
Chicks on high HUFA diets had increased immunocompetence compared to those on low HUFA diets regardless of food quantity (ANOVA: treatment $F_{3,80} = 38.187$, $p < 0.0001$; Figure 2.1e). Even Lh chicks had significantly higher PHA immune response ratios than did Ll chicks

(Table 2.3; Figure 2.1e). Among the high HUFA groups, Hh chicks had significantly higher immune response ratios than Hl chicks (Table 2.4). Among the low HUFA groups, there were no significant differences between Hl and Lh chicks (Table 2.3).

Table 2.3 Immunocompetence analysis of variance and Tukey post-hoc tests

Variable	Degrees of Freedom	F-value	P-value	Tukey Post-Hoc Test for Treatment Group
Treatment Group	3	38.187	< 0.0001	Ll < Hh (< 0.001)
Nest	8	1.891	< 0.1	Ll < Hl (< 0.001)
Residuals	30			Ll < Lh (< 0.001)
				Lh < Hh (< 0.01)
				Hl < Hh (< 0.001)
				Hl = Lh

Figure 2.1 Reaction norms for: (a) mass, (b) size-specific mass growth rate, (c) size-specific skeletal growth rate, (d) body condition, (e) immunocompetence, and (f) basal metabolic rate. Treatment means and standard error bars are shown. Treatment means and standard error bars are shown. Black represents high long-chain omega-3 polyunsaturated fatty acid (HUFA) treatments and gray represents low HUFA treatments.



Patterns in BMR were the reverse of those in immunocompetence (Kruskal Wallis: Chi-squared= 7.941, df=3, $p < 0.047$; Figure 2.1e-f). Hh chicks had the lowest basal metabolic rates (BMR) while Ll chicks had the highest BMR and these differences were significant (Table 2.4; Figure 2.1f). Hl chicks and Lh chicks had similar BMR, which were not significantly different (Table 2.4; Figure 2.1f). We also found that Ll chicks had significantly higher BMR than Hl chicks and Lh chicks had significantly higher BMR than Hh chicks (Table 2.4; Figure 2.1f).

Figure 2.2 Chick mass over time. Treatment means and standard error bars are shown. Black circles represent our high HUFA, high quantity treatment (Hh), gray circles represent our high HUFA, low quantity treatment (Hl), black triangles represent our low HUFA, high quantity treatment (Lh), and gray triangles represent our low HUFA, low quantity treatment (Ll).

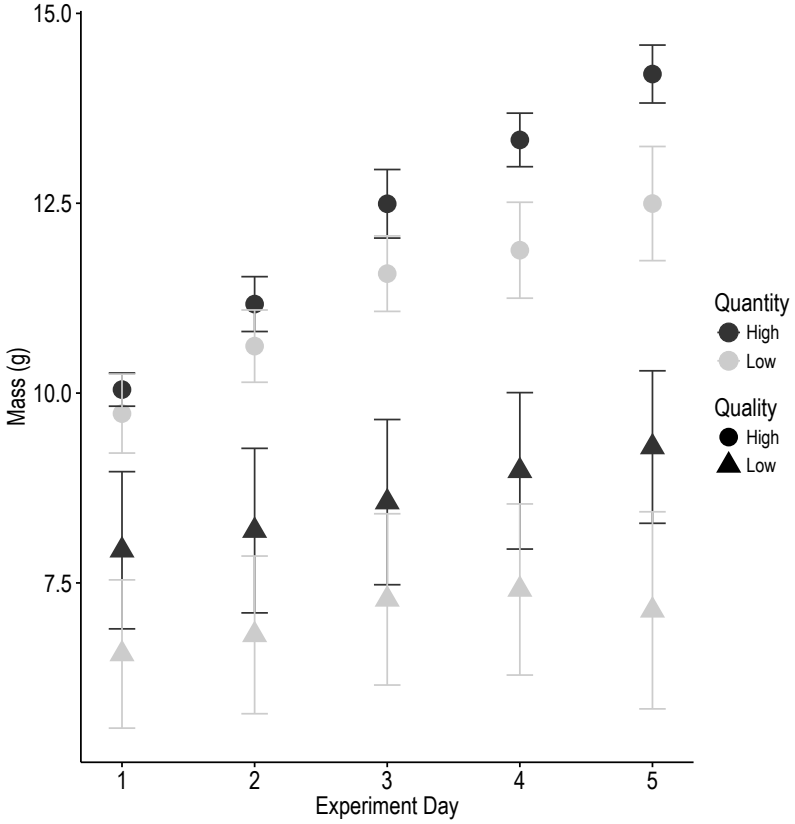


Table 2.4 Basal metabolic rate Kruskal-Wallis and Dunn tests. DF is degrees of freedom.

Mass-specific basal metabolic rates				
Variable	DF	Chi-squared Value	P-value	Dunn Post-Hoc Test
Treatment Group	3	7.941	< 0.05	Ll < Hh (< 0.01)
				Ll = Hl
				Ll < Lh (< 0.05)
				Lh = Hh
				Lh = Hl
				Hl < Hh (< 0.05)

Whole organism basal metabolic rates				
Variable	DF	Chi-squared value	P-value	Dunn Post-Hoc Test
Treatment Group	3	6.0362	0.1099	Hh < Hl (< 0.05)
				Hh = Lh
				Hh < Ll (< 0.05)
				Lh = Hl
				Ll = Ll
				Ll = Hl

Table 2.5 Fatty acid composition Kruskal-Wallis and Dunn tests

Brain EPA				
Variable	DF	Chi-squared Value	P-value	Dunn Post-Hoc Test
Treatment Group	3	7.5662	< 0.10	Ll = Hl
				Ll = Lh
				Hl = Hh
				Ll < Hh (< 0.01)
				Lh < Hh (< 0.05)
				Hl < Hh (< 0.05)

Brain DHA				
Variable	DF	Chi-squared Value	P-value	Dunn Post-Hoc Test
Treatment Group	3	8.3162	< 0.05	Ll < Hh (< 0.10)
				Ll = Hl
				Ll = Lh
				Lh < Hh (< 0.01)
				Hl = Hh
				Lh < Hl (< 0.05)

Muscle EPA				
Variable	DF	Chi-squared Value	P-value	Dunn Post-Hoc Test
Treatment Group	3	8.3162	< 0.05	Ll < Hh (< 0.1)
				Ll < Hl (< 0.05)
				Ll = Lh
				Lh < Hh (< 0.05)
				Lh < Hl (< 0.01)
				Hl = Hh

Muscle DHA

Variable	DF	Chi-squared Value	P-value	Dunn Post-Hoc Test
Treatment Group	3	3.8824	NS	Ll = Hh
				Ll < Hl (< 0.05)
				Ll = Lh
				Lh = Hh
				Lh < Hl (< 0.1)
				Hh < Hl (< 0.1)

Brain omega-3

Variable	DF	Chi-squared Value	P-value	Dunn Post-Hoc Test
Treatment Group	3	6.6397	< 0.10	Ll = Hh
				Ll = Hl
				Ll < Lh (< 0.1)
				Hl = Hh
				Hl < Lh (< 0.05)
				Hh < Lh (< 0.05)

Brain omega-6

Variable	DF	Chi-squared Value	P-value	Dunn Post-Hoc Test
Treatment Group	3	4.4559	NS	Ll < Hh (< 0.1)
				Lh = Hh
				Lh < Hl (< 0.1)
				Lh < Ll (< 0.1)
				Hl = Hh
				Hl < Lh (< 0.1)

Muscle omega-3

Variable	DF	Chi-squared Value	P-value	Dunn Post-Hoc Test
Treatment Group	3	4.1691	NS	Ll = Hh
				Ll = Hl
				Ll = Lh
				Lh = Hh
				Lh < Hl (< 0.05)
				Hl = Hh

Muscle omega-6

Variable	DF	Chi-squared Value	P-value	Dunn Post-Hoc Test
Treatment Group	3	5.4485	NS	Lh = Hh
				Lh = Hl
				Lh < Ll (< 0.1)
				Hl < Ll (< 0.05)
				Hl = Hh
				Hl < Ll (< 0.05)

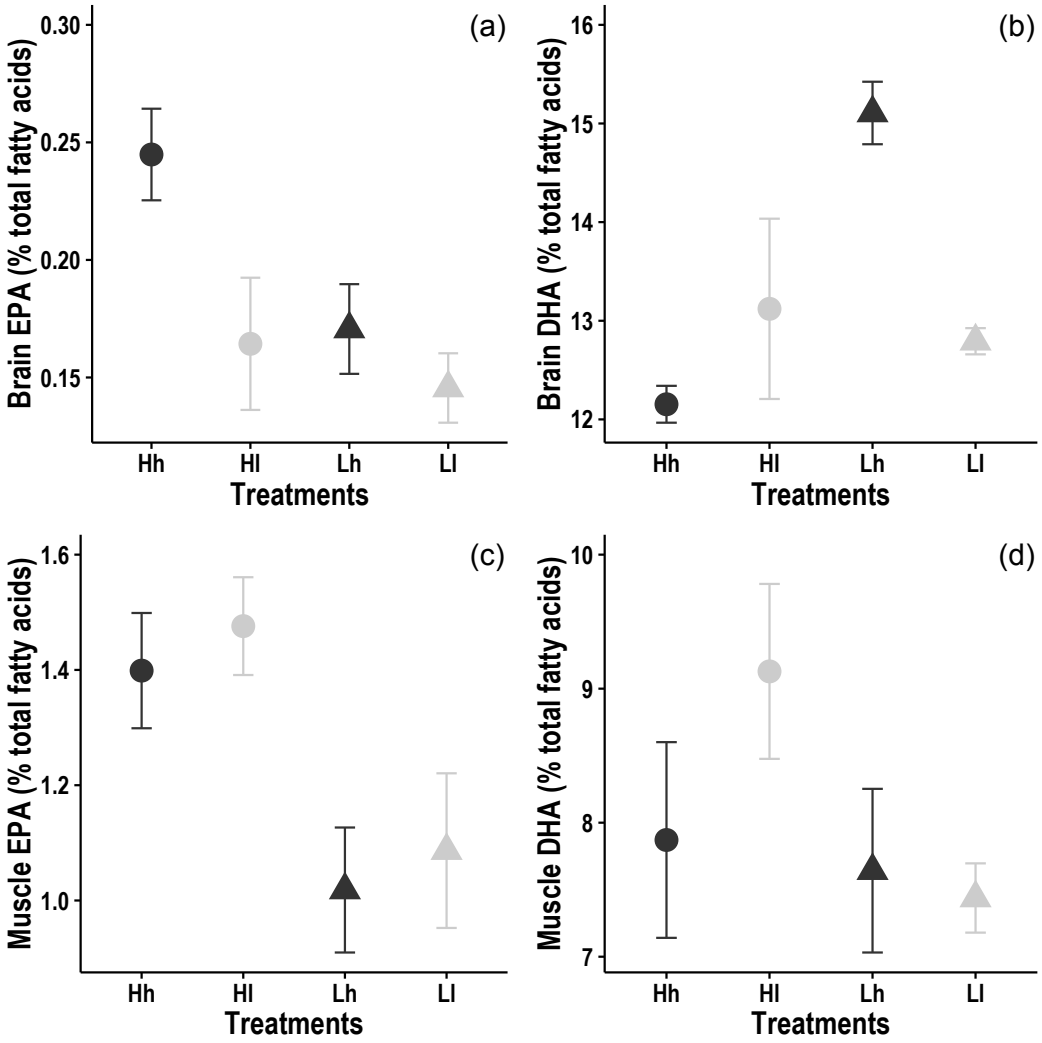
Table 2.6 Brain and muscle fatty acid composition two-sample t-tests

	EPA		
Variable	Degrees of Freedom	t-value	P-value
Tissue Type	16.117	-14.9074	< 0.001
	DHA		
Variable	Degrees of Freedom	t-value	P-value
Tissue Type	29.437	11.0112	< 0.001
	Omega-3		
Variable	Degrees of Freedom	t-value	P-value
Tissue Type	28.832	0.9059	NS
	Omega-6		
Variable	Degrees of Freedom	t-value	P-value
Tissue Type	17.092	-18.952	< 0.001

We found both treatment and tissue-based differences in fatty acid composition (Table 2.5, Table 2.6; Figure 2.3). Hh chicks had significantly higher percentages of EPA in brain while chicks on both high HUFA diets had significantly higher percentages of EPA in muscle (Kruskal Wallis for brain EPA: Chi-squared= 7.567, df=3, p = 0.056; Kruskal Wallis for muscle EPA: Chi-squared= 9.088, df=3, p = 0.028; Table 2.5; Figure 2.3). The percentage of DHA in brain was significantly higher in Lh chicks compared to chicks on either high HUFA diet (Kruskal Wallis: Chi-squared= 8.316, df=3, p = 0.040; Table 2.5; Figure 2.3b). In contrast, the percentage of DHA in muscle was highest in Hl chicks, but was only significantly higher than the percentage of DHA in muscle of Ll chicks (Kruskal Wallis for muscle DHA: Chi-squared= 3.882, df=3, p = 0.275; Table 2.5; Figure 2.3d). The percentage of total omega-3 fatty acids in brain was also significantly higher in chicks on Lh diets compared to chicks on high HUFA diets (Table 2.5). We found no significant differences in either the proportion of total omega-6 fatty acids in either brain or muscle or the percentage of total omega-3 fatty acids in muscle (Table

2.5). Muscle had significantly higher percentages of EPA and total omega-6 fatty acids compared to brain, but had similar percentages of total omega-3 fatty acids and significantly less DHA (Table 2.5).

Figure 2.3 Fatty acid composition results for: (a) brain EPA, (b) brain DHA, (c) muscle EPA, and (d) muscle DHA. Treatment means and standard error bars are shown. Black circles represent our high LCPUFA, high quantity treatment (Hh), gray circles represent our high HUFA, low quantity treatment (Hl), black triangles represent our low HUFA, high quantity treatment (Lh), and gray triangles represent our low HUFA, low quantity treatment (Ll).



Discussion

We asked if food quality, in terms of HUFAs, was as important as food quantity for a model aerial insectivore species, the Tree Swallow. We manipulated food quantity and fatty acid composition in a fully factorial design and assessed performance in Tree Swallow chicks by measuring changes in size (mass, headbill length, and tarsus length), body condition, and differences in immunocompetence and BMR at the conclusion of the experiment. Overall, we found strong evidence that HUFA content is as important, if not more important, than food quantity for aerial insectivores (Figures 2.1-2.2). We also found significant differences in the fatty acid composition of chicks on different diets, which suggested that the chicks preferentially retained EPA and DHA (Figure 2.3).

Chicks on the high HUFA, high quantity diet (Hh) grew the fastest and were in the best condition while chicks on the low LCPUFA, low quantity diet (Ll) grew the slowest and were in the poorest overall condition (Figures 2.1-2.2). Interestingly, chicks on the high HUFA, low quantity diet (Hl) performed better than did chicks on the low HUFA, high quantity diets (Lh) (Figures 2.1-2.2). Increasing the quantity of low HUFA food had no effect on growth rates or condition. Body mass and condition are two of the most important predictors of Tree Swallow fledgling success and survival in natural systems (Ardia 2005). Our results suggest that wild chicks with access to high quality food resources, such as aquatic insects, are likely to be in better condition than those with access to only lower quality terrestrial food resources. In addition, more high quality food appears to further increase body mass and condition, while more low quality food does not.

We found no significant differences in head-bill or tarsus growth rates across treatments (Figure 2.1c). This provides evidence that there is strong pressure to develop at a specific rate,

even at the cost of overall condition. Tree Swallows nestlings, like other passerines, suffer high mortality from predation (Leech and Leonard 1997) and although body mass and condition are strong predictors of survival after fledging, there appears to be greater pressure to quickly reach fledgling size to be ready to fledge if threatened by predation (Winkler 1993; Winkler and Adler 1996).

We found that food quantity and quality had significant interacting effects on immunocompetence, measured as PHA ratio, across treatments (Figure 2.1e). In birds, PHA ratio is an indicator of acquired T-cell proliferation and the ability to produce lymphocytes in response to pathogens (Stambaugh et al. 2011). We found that Hh chicks had the highest PHA ratios while Ll chicks had the lowest ratios and Hl and Lh chicks had equivalent, intermediate PHA swelling responses. Our results suggest that wild Tree Swallow chicks with access to more food, especially high quality aquatic insects containing EPA and DHA, may be more likely to mount an effective immune response (Ardia 2005, Paquette et al. 2013). In addition to predators and food deprivation, pathogens are a significant source of early mortality in nestling Tree Swallows (Duff and Ball 2002) and greater immunocompetence from higher food quantity and quality likely increases Tree Swallow chick survival.

Food quantity and quality also had significant interacting effects on BMR across treatments (Figure 2.1f). Hh chicks had the lowest metabolic rates while Ll chicks had the highest BMR, either mass corrected or whole organism (Table 2.4). Our low and high HUFA feeds had equal caloric content (Supplementary Table 2.1) so differences in BMR are likely due to effects of feed fatty acid composition and total HUFA content, not total energy. The negative relationship between total HUFA content of feed and BMR could have resulted from costs of ALA elongation and desaturation to HUFAs. For example, although feed for Hl and Hh chicks

had the same fatty acid composition, Hl chicks consumed less total HUFAs compared to Hh chicks, and thus may have required additional energy to convert ALA into HUFAs. Our findings agree with those of previous studies: for example, Pierce et al. (2005) found that increasing unsaturated fatty acids in diet decreased peak metabolic rate for Red-Eyed Vireos. Across all treatments, our findings support the inverse relationship observed by Hulbert et al. (2002) between avian body mass and both BMR and breast muscle DHA across bird species.

The fatty acid composition of chicks provided evidence for both dietary accumulation and preferential retention of the long-chain omega-3 fatty acids EPA and DHA (Figure 2.3). In brain tissue, the percentage of EPA was highest in Hh chicks, whose diet contained both the highest percentage and the greatest total amount of EPA (Figure 2.3a). Chicks on Hh and Hl diets had the highest percentages of EPA in muscle tissue (Table 2.4; Figure 2.3c). The Lh and Ll diets may not have contained sufficient amounts of EPA to accumulate dietary EPA or Tree Swallows may be inefficient at converting ALA to EPA. This suggests that EPA accumulation in the Tree Swallow tissues may be based on dietary availability of EPA, which is consistent with findings in other taxa (Newman et al. 2002).

In brain tissue, the percentage of DHA was highest in Lh chicks (Figure 2.3b). This could have stemmed from either increased elongation of ALA or preferential HUFA retention in Lh chicks. We suggest a combination of elongation and preferential retention may have been at work: Lh chicks would have had more energy to devote to elongation than did low quantity chicks and more non-HUFA fatty acids in diet to preferentially oxidize for fuel than did high HUFA chicks. Tree Swallow muscle tissue had significantly less DHA than did brain (Table 2.5; Figure 2.3), potentially because phospholipid-DHA is a key component of neural tissue (Farkas et al. 2000). In muscle tissue, the proportion of DHA in muscle was highest in Hl chicks (Figure

2.3d), which we suggest was due to preferential retention because Hl chicks were limited in energy, but not in HUFAs.

Chicks on low HUFA and/or low quantity diets may have either converted ALA to HUFAs or preferentially retained HUFAs already present in tissue. Studies suggest that chicken embryos preferentially remove HUFAs from yolk (Lin et al. 1991). However, this does not appear to be the case with altricial chicks, such as Barn Swallows (*Hirundo rustica*), which contain much less DHA at hatch than do precocial birds (Speake and Wood 2005). We were unable to control maternal fatty acid investment in eggs or parental feeding during the chicks' first few days of life, and all chicks originated from nest boxes on or near water. Thus, Hl and Lh chicks likely preferentially retained DHA from eggs and early life while oxidizing other dietary fats for energy. In contrast, Hh chicks may paradoxically have had lower tissue DHA concentrations precisely because DHA was abundant in diet, obviating the need to preferentially retain DHA.

Past work on chickens found higher levels of HUFAs in diet translated into increased proportions of HUFAs in breast muscle (e.g., Newman et al. 2002). We found that increasing the concentrations of HUFAs in diet did not necessarily result in increased HUFA content in Tree Swallow tissue. Instead, our findings on aerial insectivore chicks are closer to those of past studies on freshwater zooplankton, which have found preferential retention and bio-magnification of HUFAs compared to other fatty acids regardless of food quality (Gladyshev et al. 2011). This suggests that there is strong pressure for aerial insectivores to obtain and retain HUFAs in the face of poor conditions. Our performance data suggest that when food quantity or quality are low, saving HUFAs for future use instead of burning them as fuel may result in lower body mass and condition. Further studies employing compound-specific stable isotope tracers

(e.g., enriched $\delta^{13}C$) will be necessary to determine if Tree Swallow chicks are able to convert ALA into HUFAs and thus whether HUFAs are beneficial or absolutely essential components of diet.

Previous work on Tree Swallows has attempted to link Tree Swallow breeding season and nestling success with food availability (Dunn et al. 2011, Winkler et al. 2013). Our findings suggest that the abundance of high quality aquatic insects relative to Tree Swallow phenology may be a better predictor of breeding success than overall insect abundance. Aquatic insect abundance peaks earlier than terrestrial insect abundance (Nakano and Murakami 2001) and aquatic insects are often the only available food early in the breeding season (McCarty 1997). Total insect abundance peaks later in the breeding season, yet Tree Swallows complete laying long before peak insect abundance and their breeding success decreases with lay date (Dunn et al. 2011). Ecologists have generally interpreted these findings to indicate that laying, though earlier than peak insect abundance, places chick rearing, thought to be the most energy-demanding phase of the breeding cycle, at a time of peak food availability (Perrins 1970). Our findings suggest an alternative interpretation, that Tree Swallows, and other birds, may be under selection to time their breeding seasons when insects high in HUFA are most available.

Our findings have significant implications for aerial insectivore conservation. Most North American aerial insectivores, including the Tree Swallow, are associated with aquatic or riparian habitats (McCarty 1997). We found evidence that feed containing HUFAs representative of aquatic insects improves multiple metrics of Tree Swallow performance and that they preferentially retain these high quality fats. Our study suggests that large quantities of terrestrial insects low in HUFAs are at best no better than even small amounts of aquatic insects, even if they have high amounts of the HUFA precursor ALA. Land conservation is not enough for aerial

insectivores to survive and thrive: managers must conserve aquatic habitats that provide aerial insectivores with the highest quality HUFA-rich aquatic insects.

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Supplemental Table 2.1 Diet composition

	High HUFA, low ALA diet	Low HUFA, high ALA diet
Ingredients	Soybean Protein, Dried Egg, Chicken Meal, Menhaden Meal, Corn Starch, Menhaden Fish Oil, Soybean Oil, DL-Methionine, Dried Brewer's Yeast, Microcrystalline Cellulose, Lecithin, Aztec Marigold Extract, Wheat Flour, Xanthan Gum, Calcium Carbonate, Choline Chloride, Taurine, Lactobacillus Acidophilus, Lactobacillus Casei, Rosemary Extract, Bifidobacterium Thermophilum, Citric Acid, Enterocoddus Faecium, and Vitamin Mineral Premix	Soybean Protein, Dried Egg, Chicken Meal, Menhaden Meal, Corn Starch, Soybean Oil, Flaxseed Oil, DL-Methionine, Dried Brewer's Yeast, Microcrystalline Cellulose, Lecithin, Aztec Marigold Extract, Wheat Flour, Xanthan Gum, Calcium Carbonate, Choline Chloride, Taurine, Lactobacillus Acidophilus, Lactobacillus Casei, Rosemary Extract, Bifidobacterium Thermophilum, Citric Acid, Enterocoddus Faecium, and Vitamin Mineral Premix
Calories (± std. error)[1*]	5.98 ± 0.088	6.07 ± 0.086
Crude Fat (± std. error)[2*]	24.5 ± 0.433%	23.7 ± 0.033%
ALA[3]	1.82%	6.25%
EPA[3]	3.74%	1.47%
DHA[3]	3.44%	1.42%
Crude Fiber[2]	2.2%	2%
Neutral Detergent Fiber[2]	17.2%	11.1%
Acid Detergent Fiber[2]	10.1%	8.5%
Moisture (± std. error)[2]	7.13 ± 0.033%	7.13 ± 0.033%
Crude Protein (± std. error)[2*]	52.93 ± 0.145%	53.07 ± 0.463%
Arginine[4]	3.4%	3.4%
Cystine[4]	1.1%	1.1%
Glycine[4]	2.0%	2.0%
Histidine[4]	1.2%	1.2%
Isoleucine[4]	2.5%	2.5%
Leucine[4]	4.2%	4.2%
Lysine[4]	3.6%	3.6%
Methionine[4]	2.1%	2.1%
Phenylalanine[4]	2.6%	2.6%
Tyrosine[4]	1.8%	1.8%
Threonine[4]	2.3%	2.3%
Tryptophan[4]	0.65%	0.65%
Valine[4]	2.8%	2.8%
Taurine[4]	0.25%	0.25%
Ash[4]	< 9%	< 9%
Calcium[2]	1.05%	1.04%
Phosphorus[2]	0.75%	0.75%
Potassium[2]	0.61%	0.65%
Magnesium[2]	0.1%	0.1%
Sodium[2]	0.218%	0.226%
Chloride[4]	0.40%	0.40%
Iron (ppm)[2]	149	194
Zinc (ppm)[2]	28	30
Manganese (ppm)[2]	57	42
Copper (ppm)[2]	9	9
Iodine (ppm)[4]	1.4	1.4
Selenium (ppm)[2]	1.04	1.05
Thiamin (ppm)[4]	9	9
Riboflavin (ppm)[4]	12	12
Niacin (ppm)[4]	68	68
Pantothenic acid (ppm)[4]	26	26
Choline chloride (ppm)[4]	1710	1710
Folic acid (ppm)[4]	4.2	4.2
Pyridoxine (ppm)[4]	10	10
Biotin (ppm)[4]	1.7	1.7
Ascorbic acid (ppm)[4]	230	230
Vitamin B_{12} (µg/kg)[4]	48	48
Vitamin A (IU/kg)[4]	11,260	11,260
Vitamin D3 (IU/kg)[4]	1845	1845
Vitamin E (IU/kg)[4]	130	130
Vitamin K (ppm)[4]	6	6
Beta-carotene (ppm)[4]	0.41	0.41

[1]Measured caloric content: averages measured directly through bomb calorimetry. Units are in kilocalories per gram of dry feed. [2]Measured content. [3]Modified fatty acid composition: averages measured directly using fatty acid extraction and chromatography. [4]Unmodified dietary components: averages based on standard Mazuri nestling feed. *Not significantly different based on Kruskal-Wallis rank sum test (Calories: Chi-squared value = 2, degrees of freedom = 2, p-value = 0.368; Crude Fat: Chi-squared value = 0.5, degrees of freedom = 2, p-value = 0.479; Crude Protein: Chi-squared value = 2, degrees of freedom = 2, p-value = 0.368).

CHAPTER 3

FRESHWATER INSECTS SUBSIDIZE AN AVIAN TERRESTRIAL PREDATOR, THE EASTERN PHOEBE, WITH CRITICAL FATTY ACIDS

Abstract

Emerging freshwater insects can subsidize terrestrial predators with energetic and nutritional resources. Because past research has focused on subsidy amount, researchers have often considered small freshwater subsidies to be relatively unimportant for most terrestrial consumers. Using compound-specific stable isotopes and captive rearing experiments for Eastern Phoebes (*Sayornis phoebe*), an insectivorous bird, we show that even small subsidies can be crucial to consumers if they provide a vital source of nutrients that are scarce in recipient food webs. We demonstrate that freshwater insects are significantly enriched in highly unsaturated omega-3 fatty acids (HUFA) compared with terrestrial insects and show for the first time that freshwater insects provide insectivorous birds with a source of HUFA even when terrestrial insects dominate avian diet. Further, we show that HUFA increase chick growth rate and condition under controlled conditions, serving as key nutrients during development. Thus, even when freshwater subsidies are quantitatively small, they can have profound qualitative impacts by providing critical nutrients that are scarce in terrestrial ecosystems.

Introduction

Energy and nutrient exchanges between ecosystems, known as subsidies, can increase consumer production beyond what would be possible with internal resources alone (Polis et al. 1997). Animals that move from freshwater to terrestrial ecosystems as part of their life cycles, like emerging freshwater insects, can subsidize terrestrial predators, such as birds, by providing them with additional sources of energy and nutrients (Nakano and Murakami 2001; Baxter et al. 2005; Muehlbauer et al. 2013). Previous work on the importance of ecological subsidies has largely focused on quantifying flux sizes of energy and nutrients moving between ecosystems (Polis et al. 1997; Gratton and Vander Zanden 2009). Many subsidies, including freshwater subsidies to terrestrial ecosystems (Muehlbauer et al. 2013; Gratton and Vander Zanden 2009), are small and highly localized, often leading researchers to the conclusion that they are relatively unimportant. Nevertheless, small subsidies have the potential to be important for consumers if they are high quality and contain key nutrients that are otherwise locally scarce or absent in recipient ecosystems (Marcarelli et al. 2011).

Emerging freshwater insects have an especially high potential to provide terrestrial insectivores in riparian areas with a freshwater source of highly unsaturated omega-3 fatty acids (HUFA; Martin-Creuzburg et al. 2017; Popova et al. 2017). HUFA are physiologically important fats involved in animal nervous, hormonal, immune, and cardiovascular systems (Twining et al. 2016a). HUFA are scarce at the base of terrestrial food webs, but can be highly abundant at the base of freshwater food webs (Twining et al. 2016a). For example, while very few terrestrial vascular plants contain any HUFA, many major groups of freshwater eukaryotic algae are rich in HUFA (Twining et al. 2016a). As a consequence of these differences in fatty acid composition at the base of terrestrial and freshwater food webs, freshwater and terrestrial insects are not

nutritionally equivalent food sources even though they have similar elemental composition (Elser et al. 2000): freshwater insects have much higher amounts of the HUFA eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) than do terrestrial insects (Hixson et al. 2015).

Some animals on HUFA-poor diets, such as terrestrial vertebrate herbivores, are relatively efficient at synthesizing EPA and DHA through elongation and desaturation from the molecular precursor, the short-chain omega-3 fatty acid alpha linolenic acid (18:3n-3, ALA), while others on HUFA-rich diets, such as marine fishes and strict carnivores like cats, have effectively lost this capacity (Twining et al. 2016a). Like other taxa with high HUFA requirements, insectivorous birds in riparian zones have evolved in environments with access to HUFA-rich prey, where the selective pressure to maintain highly efficient enzymes for ALA elongation and desaturation has been low. We therefore predict that insectivores that consume freshwater prey are likely to have low capacity to synthesize HUFA from ALA and high HUFA needs. Recent work shows that diets containing freshwater insects or with HUFA supplements and can improve performance in the nestlings of insectivorous riparian birds (Twining et al. 2016b; Dodson et al. 2016), suggesting that riparian insectivorous birds may be heavily reliant upon small freshwater subsidies of nutritionally crucial, but rare nutrients. Recent work also shows that riparian insectivore nestlings have limited abilities to convert ALA into HUFA (Twining et al. 2017). Birds and other riparian insectivores that rely on freshwater insects for HUFA may be uniquely susceptible to nutritional mismatches without access to healthy aquatic ecosystems, making freshwater conservation essential for their survival.

To understand the degree to which riparian insectivorous birds rely upon freshwater versus terrestrial insects for their overall energy and nutrients, we reconstructed the diets of wild

Eastern Phoebes (*Sayornis phoebe*), a common and widespread avian insectivore (Weeks 2011), at three different sites in the Finger Lakes Region of New York State, USA. To determine where Eastern Phoebes obtain their overall diet and their HUFA in nature, we collected representative freshwater and terrestrial insects. We then analyzed bulk $\delta^{13}C$ and $\delta^{15}N$ of all samples and used stable isotope mixing-models to estimate the proportion of terrestrial versus freshwater insects in Eastern Phoebe nestling diets. To understand the nutritional significance of HUFAs on performance during development, we examined the effect of HUFAs on nestling growth and body condition through a controlled laboratory manipulation of dietary HUFA content.

Methods

To estimate the percentages of freshwater and terrestrial insects in overall Eastern Phoebe (*Sayornis phoebe*) diets, we used bulk carbon, nitrogen, and hydrogen stable isotope analyses, which allowed us to discriminate between freshwater and terrestrial dietary resources. We collected freshwater insects, terrestrial insects, and Eastern Phoebe chick blood from West Candor Creek (42.2245ºN, -76.4137ºW), Miller Creek (42.2861ºN, -76.4512ºW), and Locke Creek (42.5755ºN, -76.5293ºW) in May and June of 2015 and 2016. Freshwater insects were captured with emergence traps, terrestrial insects were captured with pan traps, and both were captured with targeted sweep netting. Freshwater insect emergence rates during the sampling period were generally low (West Candor Creek: mean of 0.11 mg m^{-2} day^{-1} ± 0.49 (1 s.d.); Miller Creek: 0.21 ± 1.21; and Locke Creek: 0.31 ± 1.64; see Chapter 4), but within the range of typical rates for temperate streams during the summer (Nakano and Murakami 2001; Baxter et al. 2004; Kraus et al. 2014; Stenroth et al. 2015). Eastern Phoebe chicks (clutch sizes of n=5, n=3, and n=4 for West Candor Creek, Miller Creek, and Locke Creek respectively) were captured and bled

under New York State Permit 1477 and United States Fish and Wildlife Service Permit MB757670. Freshwater insects included Baetidae (Ephemeroptera), Chironomidae (Nematoceran Diptera), Heptageniidae (Ephemeroptera), Anisoptera, Perlidae (Plecoptera), Tipulidae (Nematoceran Diptera), and Trichoptera. Terrestrial insects included Coleoptera, Diptera, Hymenoptera, and Lepidoptera. Samples of $n \geq 3$ insects per taxon were dried at approximately 45ºC for a minimum of 48 hours and before being ground and packed for analyses. Approximately 0.5 mg of sample was used for $\delta^{13}C$ and $\delta^{15}N$ analyses carried out at the Cornell University Stable Isotope Laboratory on a Thermo Delta V isotope ratio mass spectrometer interfaced to a NC2500 elemental analyzer. Methionine and three additional in-house standards (www.cobsil.com) were used to standardize carbon stable isotopes values to Vienna Pee Dee Belemnite (VPDB) and nitrogen stable isotope values to N_2 of atmospheric air. We also analyzed bulk $\delta^{2}H$ at the Cornell Stable Isotope Laboratory (standardized to Vienna standard mean ocean water). $\delta^{2}H$ did not offer additional discriminating power between freshwater and terrestrial sources at our sites (Figure S2) so we did not include $\delta^{2}H$ in mixing models.

We used the R package MixSIAR (Stock et al. 2016) to reconstruct Eastern Phoebe diets. Prior to running mixing models, we removed $\delta^{15}N$ terrestrial insect taxa that had substantially higher $\delta^{15}N$ values than those of Eastern Phoebes and which were unlikely to contribute substantially to Eastern Phoebe diets (*Drosophilia* spp., Staphylinidae, and Scarabidae) based on past diet studies (Weeks 2011). We ran mixing models using trophic discrimination factors (TDF) for bulk $\delta^{13}C$ and $\delta^{15}N$ (2.71 ± 0.38‰ for $\delta^{15}N$ and 0.29 ± 0.12‰ for $\delta^{13}C$ [means and standard deviations]) that we developed based on comparisons of Tree Swallow (*Tachycineta bicolor*) chick blood samples relative to known food under experimental conditions (Twining

and Shipley, personal observation). Our experimentally-estimated TDF values are within range of those of other passerines (Healy et al. 2016). We used uniformed priors in all models (i.e., we started with assumption that Eastern Phoebes consume 50% freshwater insects and 50% terrestrial insects). All models included site as a random factor, canopy cover over the stream as a continuous factor, and were run with a long model run time to reach model convergence. See supplemental material for MixSIAR diagnostic material.

To examine fatty acid composition and HUFA sources, we prepared fatty acid methyl esters from freshwater insects, specifically, Baetidae, Chironomidae, Heptageniidae, Odonata, Perlidae, Tipulidae, and Trichoptera (Hydropsychidae), and terrestrial insects, specifically, Coleoptera, Diptera, Hymenoptera and Lepidoptera. We extracted whole blood fatty acid methyl esters (FAMEs) using a modified one-step method (Garces and Mancha 1993). We quantified fatty acid composition using a BPX-70 (SGE Inc.) column and a HP5890 series II gas chromatograph-flame ionization detector (GC-FID). Chromatogram data were processed using PeakSimple. Response factors were calculated using the reference standard 462a (Nucheck prep). FAMEs were identified using a Varian Saturn 2000 ion trap with a Varian Star 3400 gas chromatography mass spectrometer run in chemical ionization mass spectrometry mode using acetonitrile as reagent gas as discussed in detail elsewhere (Van Pelt and Brenna 1999). We used gas chromatography combustion isotope ratio mass spectrometry (GCC-IRMS) to measure the $\delta^{13}C$ signatures of ALA, EPA, and DHA (Goodman and Brenna 1992; Plourde et al. 2014). An Agilent 6890 GC was interfaced to a Thermo Scientific 253 isotope-ratio mass spectrometer via a custom-built combustion interface. Peaks were confirmed to be baseline separated and were calibrated against working standards with isotope ratios traceable to international standards calibrated to VPDB (Caimi et al. 1994; Zhang et al. 1995). We tested for differences in ALA and

EPA by terrestrial or freshwater insect origin using general linear models in R (3.3.3). We did not test for differences in DHA and do not display results for DHA because we only found detectable levels of DHA in predatory stoneflies (Perlidae).

To test the effects of HUFA on Eastern Phoebe performance, we raised Eastern Phoebe chicks in the laboratory on either 1) a high omega-3 (HUFA = EPA+DHA), but low short-chain omega-3 (ALA) diet, or 2) a high ALA, low HUFA diet. We collected seventeen wild Eastern Phoebe chicks from 4 nests around Ithaca, NY under New York State Permit 1477 and United States Fish and Wildlife Service Permit MB757670. All chicks were approximately 4-5 days old and in their exponential growth stage (Murphy 1981; Murphy 1994). To minimize effects of genes and shared pre- and post-hatch environment, chicks from individual nests were sorted into each of the two diet treatments. Details on chick care and feed composition are described in (Twining et al. 2016b). We measured chick mass and headbill length and calculated specific growth rate ([ln(mass on day x) – ln(mass on day 0)] / [day x – day 0]) (Lampert and Trubeskova 1996) and body composition (mass / headbill length). We analyzed growth rates and condition in R (3.3.3) using general linear models with combinations of food treatment, nest, experiment date, and individual identity as factors (Tables 3.2-3.4). We assessed relative model support using Aikaike's Information Criterion (Burnham and Anderson 2003).

Results

We found that Eastern Phoebe chicks consumed both freshwater and terrestrial insects, but that the proportions varied substantially across the landscape (Figure 3.1A-C, Figures 3.2-3.3, Figures S3.1-3.5; Tables 3.1, S3.1). Hydrogen stable isotope values did not offer additional discrimination power beyond carbon and nitrogen (Figures 3.2-3.3; Tables 3.1). Eastern Phoebe

chicks around West Candor Creek (Figure 3.1A) consumed more freshwater than terrestrial insects: (71.8% freshwater insects, 28.2% terrestrial insects, standard deviation of 4.1%), chicks at Locke Creek (Figure 3.1B) consumed equivalent amounts of freshwater and terrestrial insects (42.1% freshwater insects, 57.9% terrestrial insects, standard deviation of 4.1%), and chicks at Miller Creek (Figure 3.1C) and consumed more terrestrial insects than freshwater insects (28.9% freshwater insects, 71.1% terrestrial insects, standard deviation of 5.7%). This highly variable dietary composition in chicks across the landscape suggests that although Eastern Phoebes are often found within foraging distance of freshwaters, they do not have strict preferences for freshwater insects and rely upon both freshwater and terrestrial resources to fulfill their high nutritional demands during growth and development.

Table 3.1 Mean and standard deviation of MixSIAR diet estimates

$\delta^{15}N$ and $\delta^{13}C$ model		
	Mean	Standard Deviation
Miller Fraction Aquatic Insects	0.29	0.06
Locke Fraction Aquatic Insects	0.42	0.21
West Candor Aquatic Insects	0.72	0.04
Miller Terrestrial Insects	0.71	0.06
Locke Terrestrial Insects	0.58	0.04
West Candor Terrestrial Insects	0.28	0.02
$\delta^{15}N$ and $\delta^{2}H$ model		
	Mean	Standard Deviation
Miller Fraction Aquatic Insects	0.33	0.07
Locke Fraction Aquatic Insects	0.36	0.03
West Candor Fraction Aquatic Insects	0.70	0.02
Miller Fraction Terrestrial Insects	0.67	0.07
Locke Fraction Terrestrial Insects	0.64	0.03
West Candor Fraction Terrestrial Insects	0.30	0.02

Figure 3.1 Percent fatty acid composition and Eastern Phoebe diet composition of freshwater (black) and terrestrial (gray) insect prey at (a) West Candor Creek, (b) Locke Creek, and (c) Miller Creek.

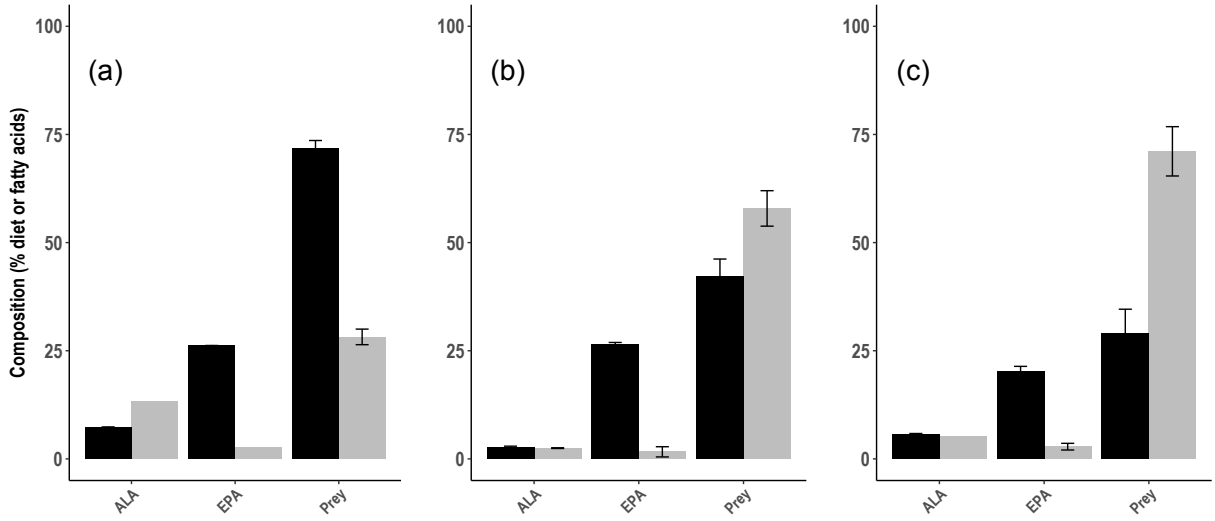


Figure 3.2 Mean and standard deviation of carbon and nitrogen bulk stable isotope values for freshwater insects (blue circles), terrestrial insects (green squares), and Eastern Phoebe (gray triangles) at (a) West Candor Creek, (b) Locke Creek, and (c) Miller Creek. Data are shown without trophic discrimination factors.

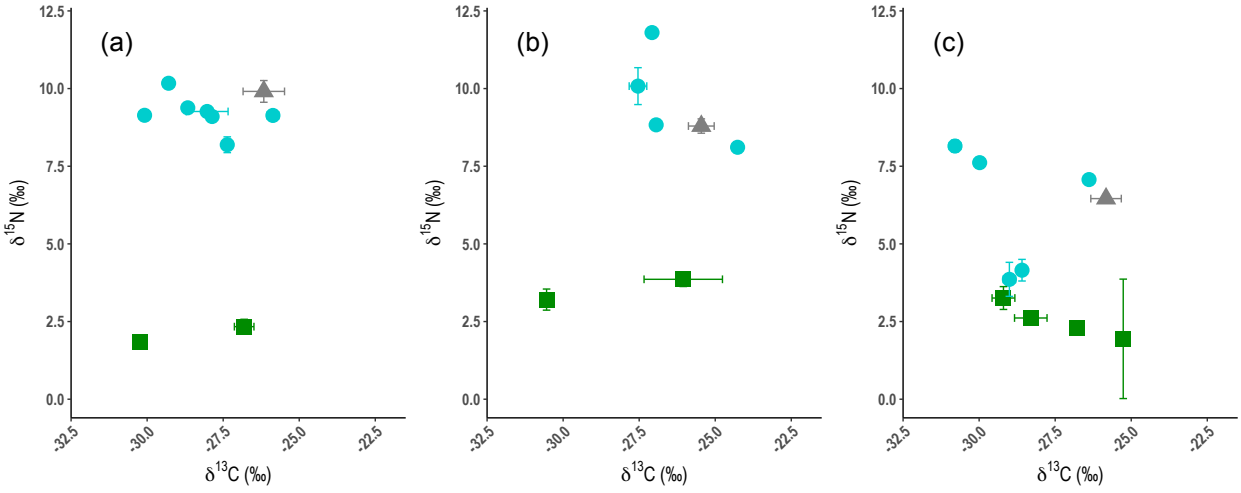


Figure 3.3 Mean and standard deviation of hydrogen and nitrogen bulk stable isotope values for freshwater insects (blue circles), terrestrial insects (green squares), and Eastern Phoebe (gray triangles) at (a) West Candor Creek, (b) Locke Creek, and (c) Miller Creek. Data are shown without trophic discrimination factors.

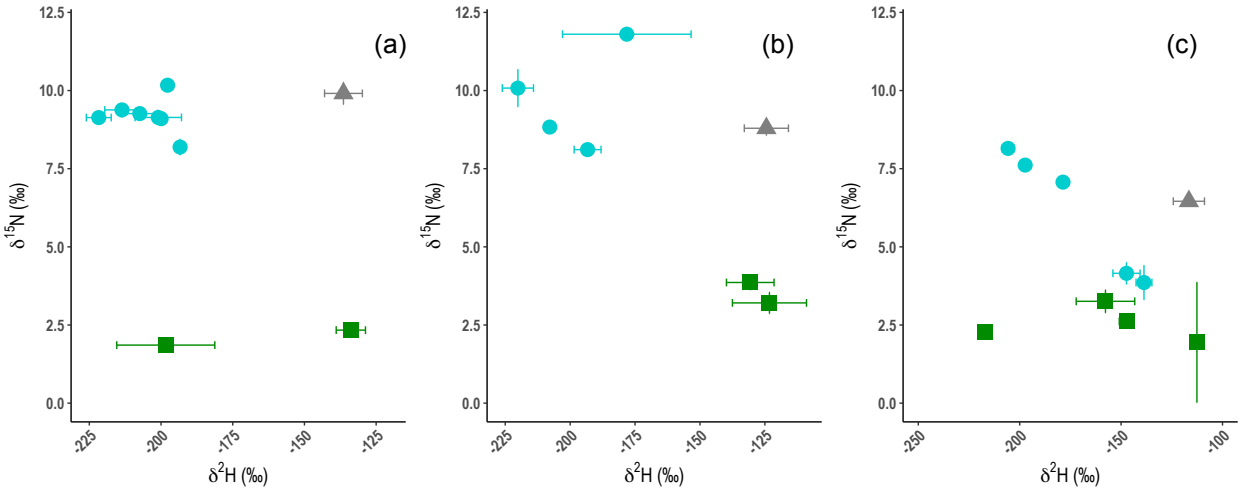

(a)
(b)
(c)
δ15N (‰)
δ2H (‰)
12.5
10.0
7.5
5.0
2.5
0.0
-225
-200
-175
-150
-125
-250
-100

Table 3.2 General linear models of ALA and EPA by insect origin (freshwater or terrestrial) for each site. SE is standard error and LS-means is least squares means.

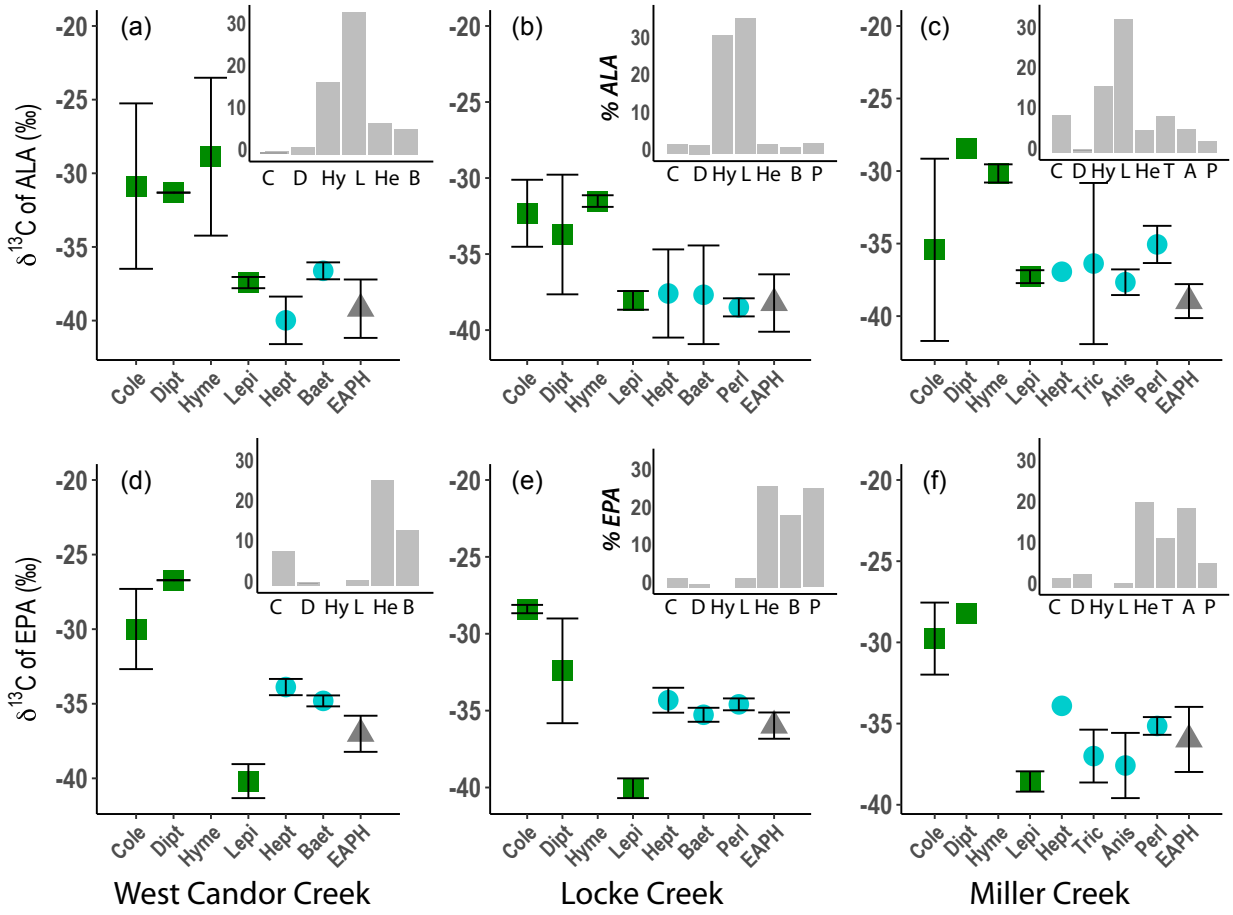
	Estimate	SE	t-value	p-value
West Candor ALA				
Intercept	7.01	6.26	1.12	0.28
Terrestrial Origin	6.32	7.23	0.87	0.40
Direction: Terrestrial ALA = Freshwater ALA				
			Null deviance: 2315.1 on 15 degrees of freedom	
			Residual deviance: 2195.2 on 14 degrees of freedom	
Locke ALA				
Intercept	2.35	4.43	0.53	0.60
Terrestrial Origin	15.86	5.86	2.71	0.01
Direction: Terrestrial ALA > Freshwater ALA				
			Null deviance: 4650.5 on 20 degrees of freedom	
			Residual deviance: 3356.8 on 19 degrees of freedom	
Miller ALA				
Intercept	5.52	2.98	1.85	0.08
Terrestrial Origin	9.83	4.32	2.28	0.03
Direction: Terrestrial ALA > Freshwater ALA				
			Null deviance: 2359.6 on 20 degrees of freedom	
			Residual deviance: 1853.6 on 19 degrees of freedom	
West Candor EPA				
Intercept	23.10	2.17	10.67	4.17e-08
Terrestrial Origin	-20.44	2.50	-8.17	1.06e-06
Direction: Terrestrial EPA < Freshwater EPA				
			Null deviance: 1515.76 on 15 degrees of freedom	
			Residual deviance: 262.53 on 14 degrees of freedom	
Locke EPA				
Intercept	24.06	0.89	26.89	< 2e-16
Terrestrial Origin	-22.61	1.18	-19.11	7.28e-14
Direction: Terrestrial EPA < Freshwater EPA				
			Null deviance: 2767.07 on 20 degrees of freedom	
			Residual deviance: 136.86 on 19 degrees of freedom	
Miller EPA				
Intercept	14.87	1.72	8.64	5.21e-08
Terrestrial Origin	-13.05	2.49	-5.24	4.70e-05
Direction: Terrestrial EPA < Freshwater EPA				
			Null deviance: 1510.70 on 20 degrees of freedom	
			Residual deviance: 618.32 on 19 degrees of freedom	

Fatty acid composition analyses allowed us to quantify nutritional differences between freshwater or terrestrial insects. Our data demonstrate that freshwater insects are significantly richer in HUFA than terrestrial insects (Figure 3.1, 3.4; Table 3.2): across the landscape, the

short-chain omega-3 HUFA precursor, ALA, was present at similar levels in both freshwater and terrestrial insects, while the HUFA EPA was significantly higher in freshwater than terrestrial insects (Figure 3.1, 3.4; Table 3.2).

Compound-specific carbon stable isotope analyses of selected fatty acids allowed us to determine if Eastern Phoebes obtained their HUFA from freshwater or terrestrial insects. Our compound-specific $\delta^{13}C$ data show that even when terrestrial resources comprise a major portion of riparian predator diets (Figure 3.1B, 3.1C), freshwater resources can provide riparian avian predators with all of their HUFA (Figure 3.4). Compound-specific $\delta^{13}C$ analyses demonstrated that Eastern Phoebe chicks received their ALA from both freshwater and terrestrial insects (Figure 3.4A-C), reflecting their mixed diet and ALA availability in both freshwater and terrestrial insects. In spite of diet variation across sites, compound-specific $\delta^{13}C$ analyses showed that Eastern Phoebe chicks at all sites derived EPA from freshwater sources (Figure 3.4D-F), reflecting the high EPA availability in freshwater insects. Terrestrial Lepidoptera compound-specific $\delta^{13}C$ values were more similar to freshwater insect $\delta^{13}C$ values (Figure 3.4), but Lepidoptera contained very minor amounts of EPA (Figure 3.4D-F), making it unlikely that Eastern Phoebes were highly reliant upon Lepidoptera or other low EPA terrestrial insects for their EPA. Thus, HUFA-rich freshwater subsidies in the form of freshwater insects are crucial to riparian predators such as Eastern Phoebes and other insectivores because freshwater insects provide a vital source of nutrients that are scarce in terrestrial systems.

Figure 3.4 Mean ALA and EPA and mean and standard deviation of compound-specific $\delta^{13}C$ at (a, d) West Candor Creek, (b, e) Locke Creek, (c, f) Miller Creek. For $\delta^{13}C$ of ALA and EPA, green squares represent whole terrestrial insects (Cole/C = Coleoptera, Dipt/D = Diptera, Hyme/Hy = Hymenoptera, Lepi/L = Lepidoptera,); blue circles represent whole freshwater insects (Anis/A = Anisoptera, Baet/B = Baetidae (Ephemeroptera), Hept/He = Heptageniidae (Ephemeroptera), Perli/P = Perlidae (Plecoptera), Tric/T = Trichoptera); and grey triangles represent Eastern Phoebe (EAPH) blood. Bars in insets represent the percentage of ALA and EPA (out of total fatty acids) in terrestrial and freshwater insects.



In addition to our finding on HUFA sources in nature, our controlled laboratory study showed that dietary HUFA increase juvenile growth rates and condition of riparian avian predators (Figure 3.5). We manipulated the HUFA and ALA content, but not the overall energetic (i.e., carbon) content, of diets for chicks in the laboratory, feeding them either: 1) a high HUFA, low ALA diet, or 2) a high ALA, low HUFA diet. HUFA levels in artificial diets fed to Eastern Phoebe chicks increased body mass, mass growth rate, and body condition (Figure 3.5). Our best-supported model for mass-specific growth rates included treatment, date of

experiment, and nest, all of which were highly significant (Figure 3.5A; $p < 0.01$; Table 3.3), while the best-supported model for condition included experiment date, treatment, the interaction of date and treatment, and nest, which were highly significant (Figure 3.5C; $p < 0.01$) as well as individual (Table 3.4). None of the factors that we included in our models had significant effects on headbill growth rate (Table 3.5), which was similar across treatments (Figure 3.5D). This suggests that riparian predators have high HUFA needs during development and that freshwater insects allow them to overcome mismatches between their nutritional needs and local terrestrial prey.

Table 3.3 General linear models for mass growth rate

Model	**AIC**		**ΔAIC**	
Treatment * Date + Nest	-172.31		0	
Treatment + Date + Nest	-171.15		1.16	
Treatment * Date + Nest + ID	-170.41		1.9	
Treatment + Date + Nest + ID	-169.25		3.06	
Treatment + Nest	-165.19		7.12	
Treatment + Nest + ID	-163.27		9.04	
Treatment + Date	-118.19		54.12	
Treatment	-117.34		54.97	
Treatment * Date	-117.24		55.07	
Treatment + ID	-115.47		56.84	
Treatment * Date + ID	-115.38		56.93	
Lowest AIC Model:	**Estimate**	**Std. Error**	**t-value**	**p-value**
Intercept	0.56	0.07	8.23	< 0.0001
Treatment	-0.19	0.05	-4.04	< 0.0005
Date	-0.05	0.02	-2.57	< 0.05
Nest	-0.05	0.01	-9.74	< 0.0001

Treatment * Date	0.03	0.01	1.71	< 0.10
Null deviance	0.39 on 50 degrees of freedom			
Residual deviance	0.08 on 46 degrees of freedom			

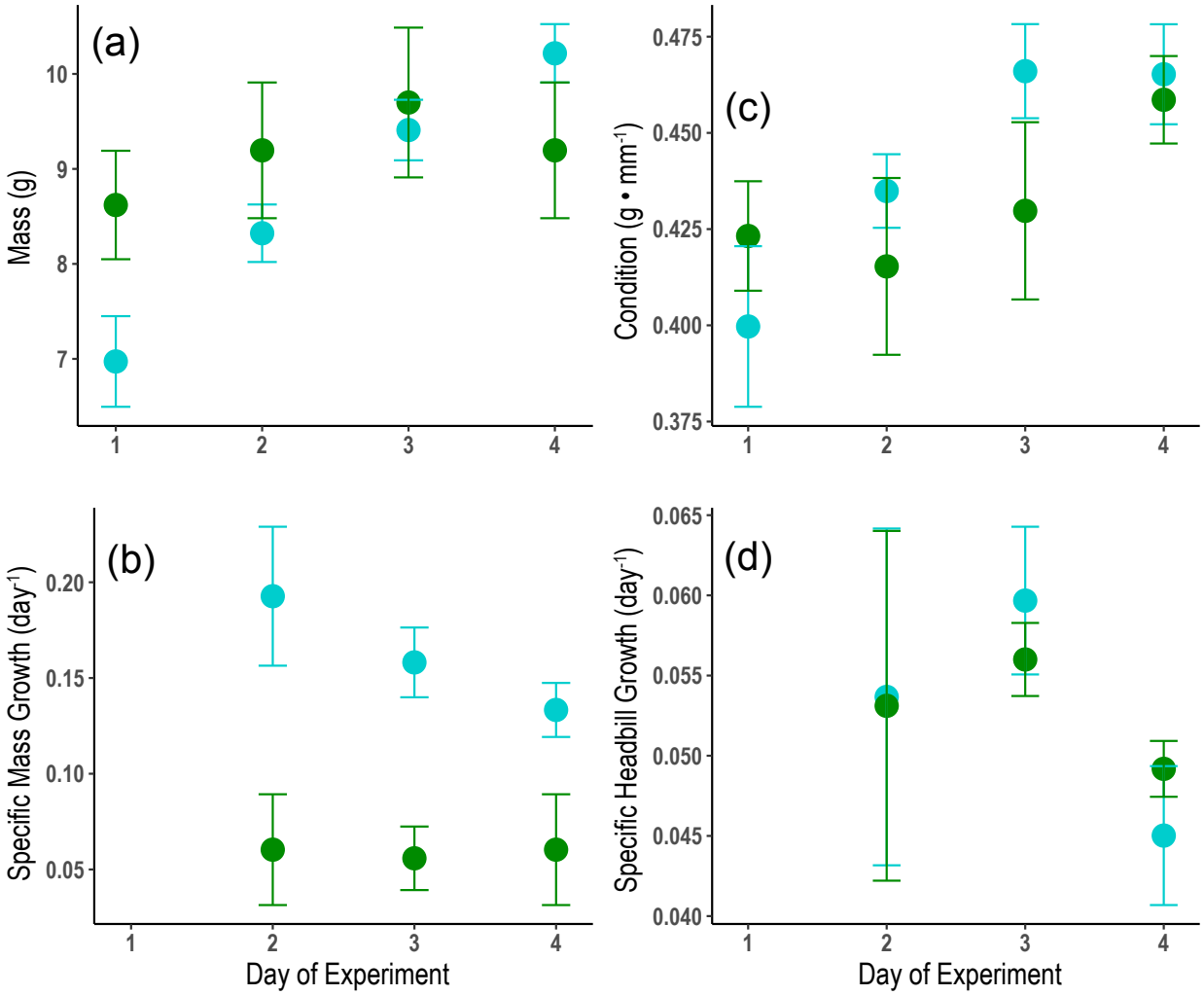
Table 3.4 General linear models for condition (mass / headbill length)

Model	**AIC**		**ΔAIC**	
Treatment * Date + Nest + ID	-169.01		0	
Treatment * Date + Nest	-168.47		0.54	
Treatment + Date + Nest + ID	-166.2		2.81	
Treatment + Date + Nest	-165.94		3.07	
Treatment * Date	-158		11.01	
Treatment + Date	-156.64		12.37	
Treatment * Date + ID	-156.46		12.55	
Treatment + Nest	-152.67		16.34	
Treatment + Nest + ID	-152.2		16.81	
Treatment	-146.62		22.39	
Treatment + ID	-144.93		24.08	
Lowest AIC Model:	**Estimate**	**Std. Error**	**t-value**	**p-value**
Intercept	0.08	0.07	1.09	0.28
Treatment	0.13	0.03	4.39	< 0.001
Date	0.04	0.03	3.40	< 0.01
Nest	0.05	0.01	3.86	< 0.001
ID	0.002	0.001	1.49	0.14
Treatment * Date	-0.02	0.01	-2.08	< 0.05
Null deviance	0.05 on 39 degrees of freedom			
Residual deviance	0.02 on 34 degrees of freedom			

Table 3.5 General linear models for headbill growth rate

Model	**AIC**		**ΔAIC**	
Treatment	-182.23		0	
Treatment + Date	-181.21		1.02	
Treatment + Nest	-180.53		1.70	
Treatment + Date + Nest	-179.67		2.56	
Treatment * Date	-179.37		2.86	
Treatment + Nest + ID	-179.25		2.98	
Treatment + Date + Nest + ID	-178.44		3.79	
Treatment * Date + ID	-177.89		4.34	
Treatment * Date + Nest	-177.76		4.47	
Treatment * Date + Nest + ID	-176.53		5.70	
Treatment + ID	-150.62		31.61	
Lowest AIC Model:	**Estimate**	**Std. Error**	**t-value**	**p-value**
Intercept	0.05	0.008	6.25	<0.0001
Treatment	0.0006	0.005	0.11	0.91
Null deviance	0.01 on 33 degrees of freedom			
Residual deviance	0.01 on 32 degrees of freedom			

Figure 3.5 Eastern Phoebe chick (a) mass, (b) mass growth rates, (c) body condition (mass divided by headbill length), and (d) headbill growth rate. Means and standard errors shown. High HUFA treatments are blue and low HUFA treatments are green.



Discussion

We asked if small ecological subsidies could have important ecological impacts on animals in recipient ecosystems by providing a source of critical, locally scarce nutrients. We investigated the importance of small freshwater subsidies of HUFA during the breeding season to Eastern Phoebes, an avian terrestrial predator, in natural riparian ecosystems as well as the effects of HUFA on developmental performance in Eastern Phoebe chicks in a controlled laboratory experiment. Overall, we show that freshwater insects in nature are substantially and significantly richer in HUFA are terrestrial insects and that emergent freshwater insects provided HUFA for Eastern Phoebe chicks across natural ecosystems. In addition, we demonstrate that HUFA have important, significant effects on growth and condition during Eastern Phoebe rapid development, echoing our previous findings in Tree Swallow (*Tachycineta bicolor*) nestlings (Twining et al. 2016b), which are also riparian aerial insectivores, relying upon similar prey base to Eastern Phoebes. Together, these findings suggest that terrestrial predators, such as riparian birds, with access HUFA-rich prey in nature, such as freshwater insects, may be highly reliant upon high nutritional quality subsidies from freshwater ecosystems even when these subsidies are small.

Our data demonstrate that freshwater insects are significantly richer in HUFA than are terrestrial insects, some of which are richer in ALA, the HUFA precursor (Figure 3.4; Table 3.2). These substantial differences in HUFA content between freshwater and terrestrial insects are consistent with the stark dichotomy in HUFA content at the base freshwater and terrestrial food webs between many freshwater algae, which are HUFA-rich and most terrestrial vascular plants, which contain only ALA (Hixson et al. 2015; Twining et al. 2016). Moreover, our data show that

even when terrestrial insects comprised a major portion of overall Eastern Phoebe chick diets (Figure 3.1B-C), freshwater insects subsidized chicks with all of their HUFA (Figure 3.4). In addition to highlighting the importance of freshwater subsidies as a way to overcome local nutritional mismatch with terrestrial resources, this suggests that chicks likely selectively retain HUFA directly from small contributions of freshwater resources rather than obtaining them from ALA in terrestrial sources.

Eastern Phoebes and other terrestrial avian insectivores in riparian areas could potentially derive their HUFAs from the short-chain omega-3 precursor ALA, which we found in terrestrial insects, especially pollinators like Lepidoptera and Hymenoptera, as well as freshwater insects. However, our laboratory results show that ALA without sufficient amounts of HUFAs does not satisfy Eastern Phoebe nutritional needs during development (Figure 3.5). ALA conversion to EPA through elongation and desaturation is an energetically demanding process and HUFA from freshwater insects present in the riparian zone provide a direct, cost-effective route for HUFA acquisition. Riparian avian insectivores like Eastern Phoebes are thus unlikely to rely upon, or be optimally efficient at, this process because they have evolved in habitats with access to HUFA-rich freshwater insects. For example, ALA to HUFA conversion efficiency in Tree Swallow nestlings, while within the low range of conversion efficiencies reported for humans (Burdge et al. 2002), is likely insufficient relative to nestling HUFA demand (Twining et al. 2017). Our findings in riparian insectivores are consistent with studies that have found limited conversion ability relative to HUFA needs in other animals like marine fishes that live in HUFA-rich environments (Twining et al. 2016a).

In addition to our data illuminating relative food quality and HUFA pathways in natural riparian systems, data from our controlled laboratory study also demonstrate that dietary HUFA

increase growth and condition in Eastern Phoebe chicks (Figure 3.5B-C). We found that dietary HUFA, but not ALA, increased mass growth rates and body condition (Figure 3.5B-C) for chicks undergoing rapid development, suggesting that riparian predators have high HUFA needs during development. As in our previous study on Tree Swallow chicks (Twining et al. 2016b), we found that dietary HUFA did not impact skeletal growth rates (Figure 3.5D), which are thought to be relatively invariant in spite of dietary variation during development in many small passerine species during development (Leech and Leonard 1997). In contrast, nestling body mass and body condition are both highly flexible based on diet and are key predictors of survival to fledge and overall breeding success (Winkler 1993; Winkler and Adler 1996). Thus, our work shows HUFA-rich freshwater subsidies in the form of freshwater insects can be crucial to riparian predators such as Eastern Phoebes and other insectivores because freshwater insects provide a vital source of nutrients that are scarce in terrestrial systems, allowing riparian predators to overcome the mismatches between their nutritional needs and local terrestrial prey.

Although it is well established that freshwater insects can subsidize riparian predators, previous studies have generally focused on subsidy quantity (Nakano and Murakami 2001, Baxter et al. 2005) while ignoring differences in the nutritional quality of freshwater and terrestrial insects (but see Martin-Creuzburg et al. 2017; Popova et al. 2017). We argue that the quality of subsidies is an important overlooked factor that must be considered in evaluating the importance of subsidies, especially when nutritional mismatches between consumers and local resources create the potential for subsidies to satisfy a key nutritional need. We show that even when freshwater insects do not dominate riparian predator diets, they have profound impacts on terrestrial food webs as nutritional subsidies and are thus a much more important ecological force than previously acknowledged. Previous researchers have argued that freshwater habitats serve

as important indicators of terrestrial ecosystem health by concentrating flows of nutrients and pollutants from the surrounding landscape (Hynes 1975; Allan 2004) and have demonstrated that healthy freshwater ecosystems provide humans with numerous highly valuable ecosystem services (Sweeney et al. 2004; Dudgeon et al. 2006). Our findings reveal the crucial nutritional support that healthy freshwater ecosystems, even when small, provide for animals in surrounding terrestrial food webs.

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Table S3.1 MixSIAR Diagnostics for $\delta^{15}N$ and $\delta^{13}C$ model

Diagnostic	Result
Gelman-Rubin	Out of 22 variables: 3 > 1.01, 0 > 1.05, 0 > 1.1
Geweke	Number of variables outside +/-1.96 in each chain: Chain 1 = 4, Chain 2 = 5, Chain 3 = 2

Figure S1.1 Pairs plots for all sites for MixSIAR $\delta^{15}N$ and $\delta^{13}C$ model

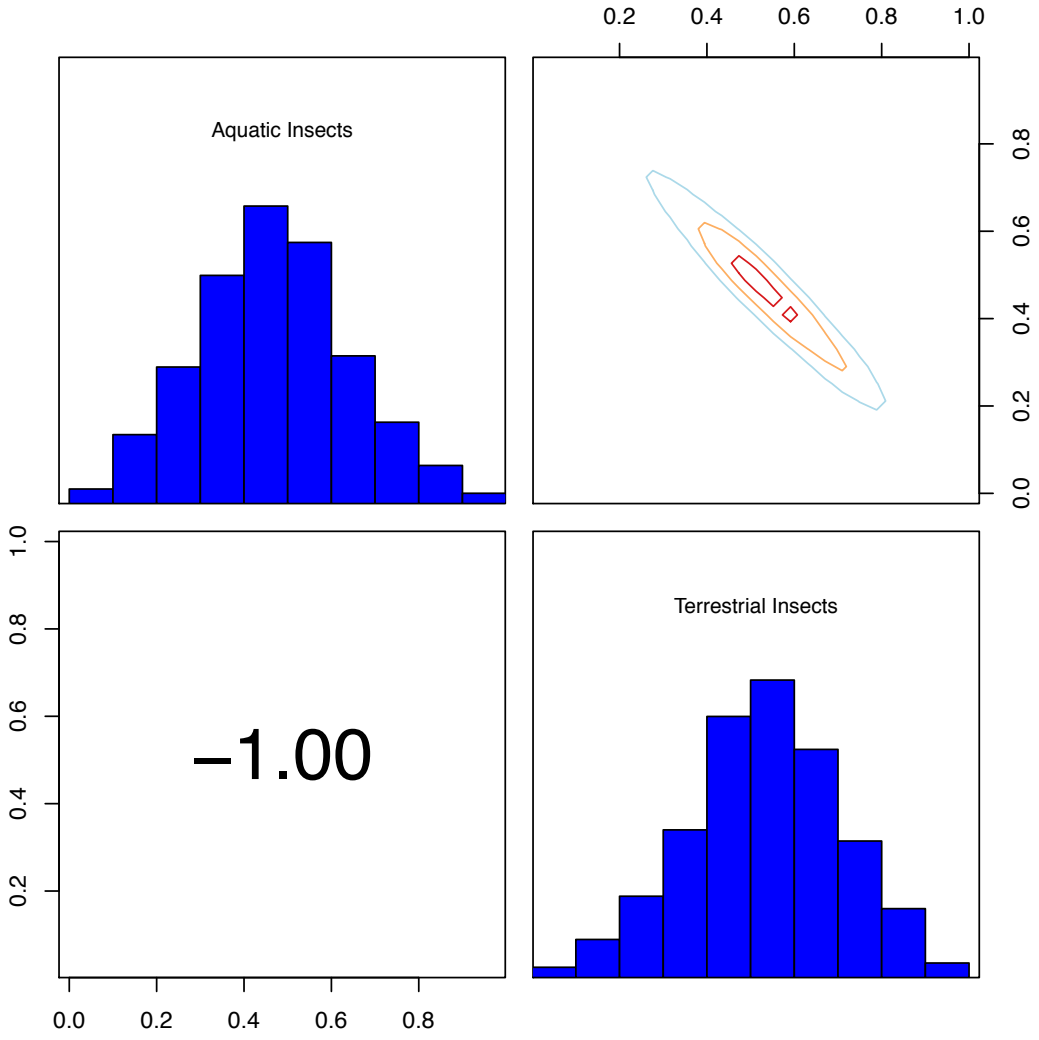


Figure S3.2 Posterior density plot for Miller Creek for $\delta^{15}N$ and $\delta^{13}C$ model

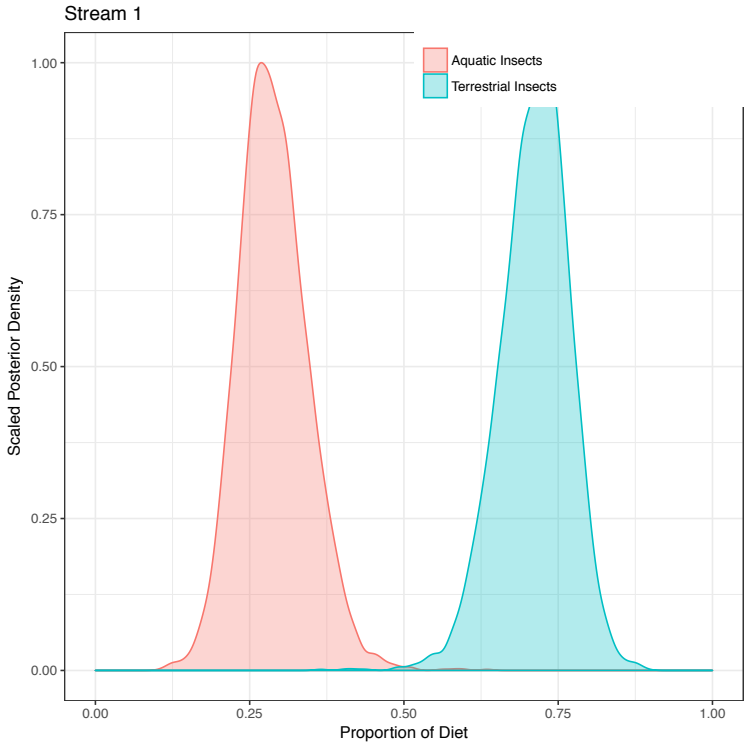


Figure S3.3 Posterior density plot for West Candor Creek for $\delta^{15}N$ and $\delta^{13}C$ model

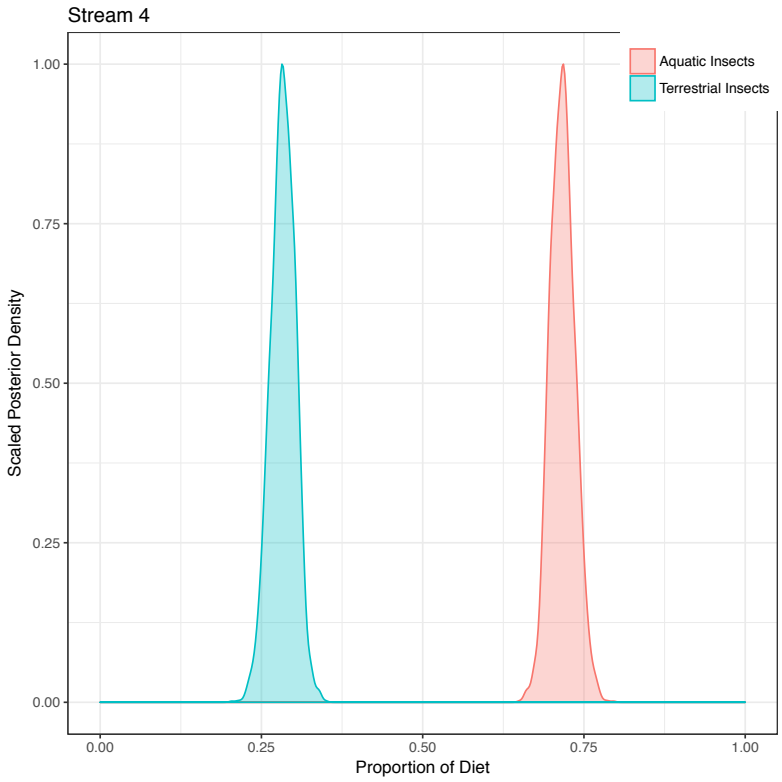


Figure S3.4 Posterior density plot of Locke Creek for $\delta^{15}N$ and $\delta^{13}C$ model

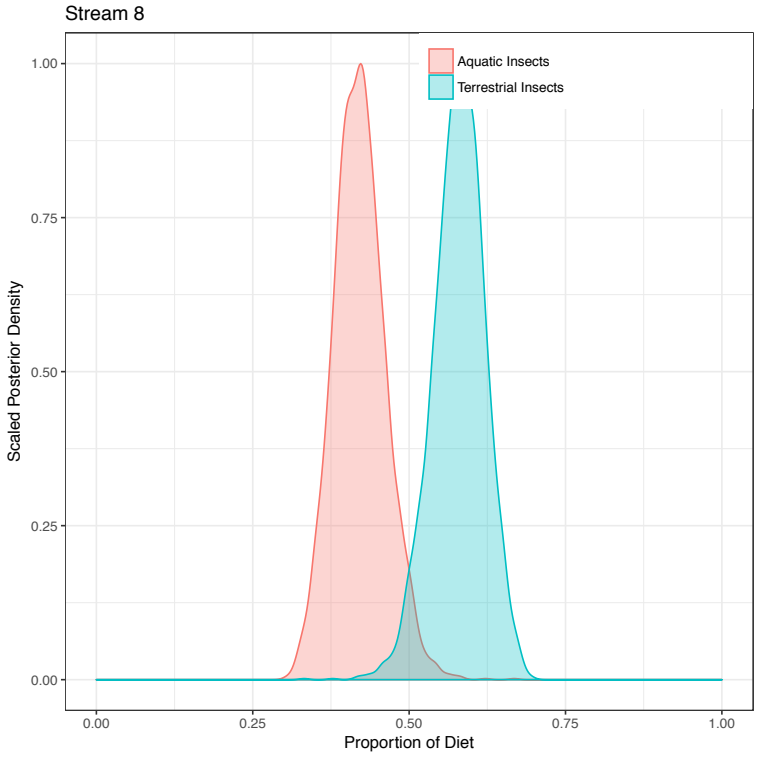
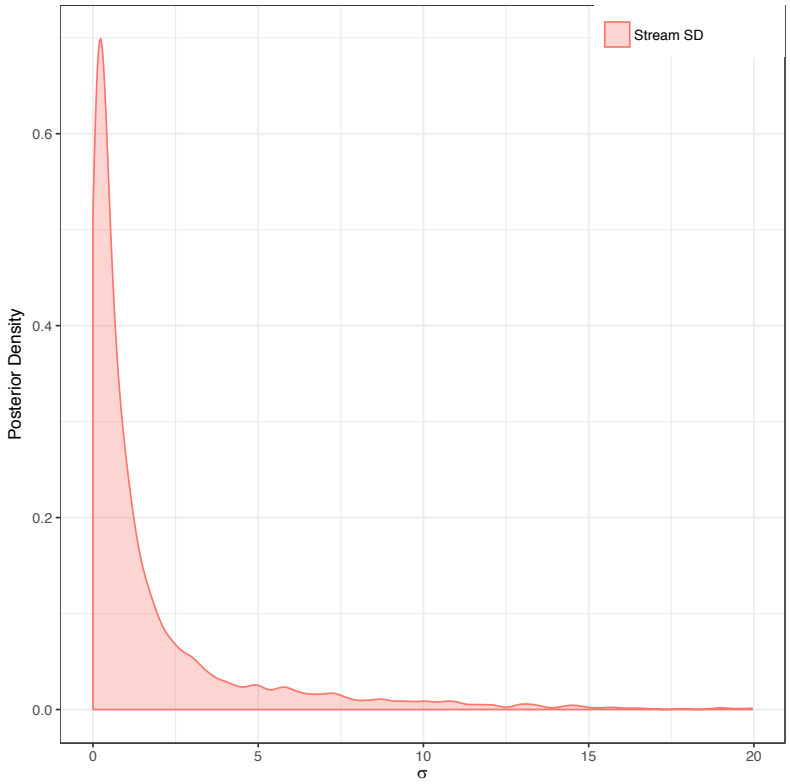


Figure S3.5 Posterior density plot of standard deviations for $\delta^{15}N$ and $\delta^{13}C$ model



CHAPTER 4

EMERGING FRESHWATER INSECTS AS SUBSIDIES TO RIPARIAN BIRDS: THE IMPORTANCE OF OMEGA-3 HIGHLY UNSATURATED FATTY ACIDS

Abstract

Small inputs of physiologically important nutritional resources have the potential to subsidize recipient ecosystems where they are locally scarce, connecting food webs at the landscape scale. While riparian predators such as insectivorous birds consume local terrestrial prey, fluxes of energy and nutrients from emergent freshwater insects can also subsidize riparian insectivores. Based on previously documented differences in fatty acid composition at the base of aquatic and terrestrial food webs, we hypothesized that freshwater resources could be important sources of omega-3 highly unsaturated fatty acids (HUFAs) for riparian consumers. Across a diverse agricultural and forested landscape, we found freshwater insects were significantly richer in HUFAs, especially eicosapentaenoic acid (EPA), while terrestrial insects had significantly higher percentages of the less bioactive HUFA precursor, alpha linolenic acid. We also found that emergent freshwater insects can provide a source of EPA for an insectivorous bird, the Eastern Phoebe (*Sayornis phoebe*). We found that freshwater insects composed a minimum of 25% of Eastern Phoebe chick diet across the landscape, likely allowing chicks to satisfy their nutritional needs. While Eastern Phoebe chicks relied on freshwater insects for up to 86% of their overall diet at some sites, there was little evidence that site-specific factors including prey availability or fatty acid composition, canopy cover, and land use explained the strength of freshwater subsidies to chicks across sites. Our results suggest that when there are major differences between the quality of local resources and the quality of subsidies, the nutritional composition of subsidies rather than their size alone may drive consumer interactions across ecosystems.

Introduction

Resource subsidies connect food webs at the landscape scale through the movement of energy and nutrients from donor to recipient ecosystems (Polis et al. 1997). Large subsidies can link ecosystems at the regional scale, such as marine subsidies from anadromous fishes that swim hundreds of miles to their freshwater spawning grounds (Durbin et al. 1979; Schindler et al. 2003; Flecker et al. 2010). Subsidies can also be much smaller and highly local, such as seasonal fluxes of terrestrial arthropods that fuel fish production in forested stream food webs (Nakano et al. 1999; Kawaguchi et al. 2003). Previous research has largely focused on quantifying fluxes of energy and nutrients in the form of biomass moving from more productive donor ecosystems to less productive recipient systems (Polis et al. 1997). However, energy and nutrient fluxes from less productive ecosystems, especially those that result from animal migration and dispersal, can also subsidize more productive ecosystems (Baxter et al. 2005; Marcarelli et al. 2011). Even small subsidies from less productive systems may have large effects on highly productive recipient systems by providing locally scarce forms of energy or nutrients.

Freshwater insects emerging from streams and lakes provide a subsidy to the consumers in the surrounding riparian zone such spiders, birds, and bats (Nakano and Murakami 2001; Baxter et al. 2005; Clare et al. 2011; Kautza and Sullivan 2016). While some freshwater systems provide exceptionally large subsidies to terrestrial systems (Dreyer et al. 2015; Gratton et al. 2017), subsidies from freshwaters like small ponds and streams are often minor compared to available terrestrial resources and are frequently highly pulsed and localized (Baxter et al. 2005; Gratton and Vander Zanden 2009; Muelbauer et al. 2013). At the landscape scale, the potential for freshwater systems to subsidize riparian consumers may be related to factors including prey availability and nutritional quality, consumer foraging preferences, and environmental factors

like local land use. For example, local land use may impact the relative availability and species composition of freshwater (Stenroth et al. 2014) and terrestrial prey (Kautza and Sullivan 2015) as well as the foraging behavior of riparian consumers like birds (Nakano and Murakami 2001; Uesugi and Murakami 2007).

Differences in the nutritional quality of freshwater and terrestrial prey and the nutritional needs of riparian consumers may also influence the importance of freshwater subsidies. Omega-3 highly unsaturated fatty acids (HUFAs), in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are especially important organic compounds (Jump 2002; Brenna and Carlson 2014) that have a high potential to subsidize riparian animals (Twining et al. 2016a). Most animals cannot synthesize HUFAs *de novo* and must either obtain HUFAs or their molecular precursor, the shorter-chain omega-3 fatty acid alpha linolenic acid (ALA), directly from their diet to survive and avoid growth limitation (Brenna et al. 2009; Twining et al. 2016a). However, HUFAs themselves are ecologically scarce and are not homogeneously distributed across the landscape. Few vascular terrestrial plants contain any detectable HUFAs, in contrast to many marine and freshwater primary producers, which contain high levels of HUFAs (Hixson et al. 2015; Twining et al. 2016a). While this dichotomy generally holds based on habitat, it is primarily based on phylogeny in that mosses are often rich in EPA while vascular freshwater plants contain only ALA (Twining et al. 2016a). Differences in the fatty acid composition between terrestrial and freshwater resources appear to persist even at higher trophic levels (Hixson et al. 2015) because the fatty acid composition of animals is highly dependent upon the fatty acid composition of their food sources (Torres-Ruiz et al. 2010; Bayes et al. 2014).

HUFA requirements may guide nutrient movement both within (Danielsdottir et al. 2007; Gladyshev et al. 2012) and across ecosystems where major disparities in HUFA availability

occur (Gladyshev et al. 2013). Streams are likely to serve as hotspots for freshwater HUFA fluxes to animals in recipient riparian terrestrial food webs because streams have high perimeter to area ratios enhancing connections with the riparian zone (Gratton and Vander Zanden 2009) and because terrestrial leaves and arthropods contain little to no HUFAs while emergent freshwater insects are rich in HUFAs, especially EPA (Hixson et al. 2015; Twining et al. 2016a). In addition, animals from other environments rich in HUFAs, including most marine fishes (Sargent et al. 1999), have lost the ability to convert ALA into HUFAs and must obtain HUFAs directly from diet while a diversity of terrestrial herbivores and omnivores appear to be relatively efficient at deriving HUFAs from ALA (Blomquist et al. 1991; Jakobsson et al. 2006). Recent research suggests that insectivorous riparian birds may require HUFAs from freshwater insects for optimal developmental performance (Twining et al. 2016b; Dodson et al. 2016; Twining et 2018).

The importance of stream HUFA subsidies to animals like birds in recipient terrestrial systems is likely determined by a combination of factors including: 1) nutritional physiology, 2) prey availability, 3) prey food quality in terms of fatty acid composition, 4) preferred avian foraging habitat, and 5) environmental factors. In this study, we asked which of these factors determine the importance of freshwater subsidies in a representative riparian insectivore, the Eastern Phoebe (*Sayornis phoebe*), across a complex forested and agricultural landscape. We sampled freshwater and terrestrial arthropod prey availability and quality, as well as Eastern Phoebe chick diets at eight stream locations to test the following two hypotheses: 1) physiological species-level nutritional requirements create demand for freshwater HUFA subsidies across the landscape, or 2) local factors including the relative availability and quality (fatty acid composition) of prey as well as environmental factors such as stream canopy cover

and land use impact the strength of freshwater subsidies to riparian predators. Based on stark differences in HUFA availability at the base of aquatic and terrestrial food webs (Twining et al. 2016a), we hypothesized that across our sites, freshwater insects would have higher percentage of fatty acids as HUFAs than would terrestrial insects, allowing them to subsidize riparian consumers. If nutritional requirements for HUFAs determine dietary patterns across the landscape, Eastern Phoebe should consume at least some proportion of freshwater insects across all sites in order to satisfy their nutritional demands. If local differences in prey quality determine overall diet and foraging strategy, then Eastern Phoebes should consume more freshwater prey at sites with lower quality terrestrial and freshwater prey. In contrast, if local differences in prey quantity determine diet, then Eastern Phoebes should consume more freshwater prey at sites with a higher relative availability of freshwater prey or a lower relative availability of terrestrial insects. Finally, if local environmental factors other than prey availability and quality, such as canopy cover, drive foraging, then subsidies to Eastern Phoebes should be unrelated either to nutritional needs or to local prey availability or quality.

Methods

In order to understand which local factors influence freshwater subsidies at a landscape level, we examined freshwater HUFA subsidies to Eastern Phoebe nestlings at eight stream and riparian sites near Ithaca, NY, USA. We first characterized environmental variables at each site including riparian land use (Table 1). Second, we sampled freshwater and terrestrial arthropod prey for riparian insectivores, quantifying relative availability and flux rates across site and collecting insect samples for analyses. Third, we sampled blood from Eastern Phoebe chicks and quantified adult Eastern Phoebe foraging patterns. Next, we performed fatty acid composition, elemental,

and stable isotope analyses on Eastern Phoebe and insect samples. We then used stable isotope mixing models to estimate the contribution of freshwater and terrestrial insects to Eastern Phoebe chick diets. Finally, we performed statistical analyses to relate prey availability, quality, and local environmental factors to Eastern Phoebe dietary patterns across the landscape.

Study site characterization

We characterized each site in terms of: land use, stream canopy cover, stream wetted width, stream discharge, mean air temperature during the sampling period, coefficient of variation in air temperature, mean stream temperature during the sampling period, coefficient of variation in stream temperature, mean stream dissolved oxygen, coefficient of variation in dissolved oxygen, stream pH, stream conductivity, mean per area chlorophyll *a*, epilithon ash free dry mass (AFDM), mean stream total phosphorus (TP) and soluble reactive phosphorus (SRP) concentrations.

To assess effects of land use on freshwater HUFA subsidies, we quantified land use using Python 3.4.3 geoprocessing tools in an Arcmap 10.3.1 (ESRI, Redlands, CA) environment. Using a 10 m digital elevation model from the USGS National Elevation Dataset Watershed, we delineated boundaries for each stream. We quantified agricultural land use using two metrics to represent the extent of agricultural land use within Eastern Phoebe foraging range: 1) agricultural land use within a 25 m buffer along a 100 m reach surrounding our study site, and 2) within a 100 m radius of the of study site. For percent agriculture within a 25 m buffer along a 100 m reach (hereafter, 25 m buffer), we created spatial buffers around each stream at a distance of 25 m using stream vector data from the New York State Department of Environmental Conservation at a 1:24,000 scale. We calculated percent agriculture within a 100 m radius of each stream by

creating a polygon that included land upstream of the study site. At each site, we also quantified percent canopy cover over the stream site using a densitometer. We measured wetted width at each site and calculated cross sectional discharge with a Marsh McBirney Flo-Mate 2000 Flow Meter (Hach Flow, Loveland, CO).

At each site, we deployed both aerial and in-stream HOBO loggers (Onset, Bourne, MA) to record temperature and light intensity during our entire sampling period. We also deployed in-stream MiniDOT loggers (PME, Vista, CA) to record dissolved oxygen (DO) concentrations for a minimum of two sets of one week intervals per site, rotating loggers between sites. We also took point measurements of stream pH and conductivity using a YSI 556 multi-probe meter (YSI Inc., Yellow Springs, OH). To quantify mean chlorophyll *a* and ash free dry mass, we removed epilithon from rocks from three locations throughout the study reach. We filtered three replicates of one to five mL of epilithon slurry from each rock section onto pre-weighed, pre-combusted 47-mm glass-fiber filters (Pall Gelman, Port Washington, New York) to measure AFDM and chlorophyll *a*. We dried filters and weighed them to estimate dry mass, combusted the filters at 500°C for ≥1 h, reweighed the filters, and subtracted the difference to obtain AFDM. We measured chlorophyll *a* content by fluorometry on a TD-700 fluorometer (Turner Designs, Sunnyvale, California). We extracted chlorophyll from samples with 25 mL of 90% ethanol for 24 h prior to analysis. We measured phosphorus concentrations (TP and SRP) via spectrophotometry with acid molybdate–antimony and ascorbic acid reagent on a Shimadzu UV mini 1240 spectrophotometer (Shimadzu Scientific Instruments, Columbia, Maryland) following digestion with potassium persulfate (Parsons et al. 1984).

Arthropod sampling

We collected freshwater insects with emergence traps and terrestrial insects as well as spiders with terrestrial pan traps. Between 17-May-2015 to 25-July-2015, we collected arthropods from traps for analyses of stable isotopes, fatty acid composition, and biomass. We deployed three traps of each type at each site. However, due to high flow events during rainstorms and riparian and in-stream large livestock movements, which caused traps to tip over, we were only able to collect emergent and terrestrial insects from one to two traps at some sites on some dates. Additional insects were captured for stable isotope analyses with targeted sweep netting in May-June 2016. We constructed 0.25 m^2 freshwater insect emergence traps with Skeeta no-seem-um netting (100 openings / cm^2; Skeeta, Brandenton, FL). Modified Starbar ® Trap n' Toss ™ (Central Life Sciences, Shaumburg, IL) disposable fly trap chambers sat atop traps to collect emerging insects. We filled the insect trap chambers with NaCl saturated stream water to preserve insects. Terrestrial pan traps for collecting arthropods consisted of containers with opening size of 0.122 m^2. We filled pan traps with soapy water and placed them 0-5 m inland from the stream wetted channel (depending on bank stability in the riparian zone) within the same reach of stream as the emergence traps.

We collected arthropods for biomass from both freshwater and terrestrial traps on the same day per site weekly during the Eastern Phoebe chick nesting period from mid-May through June. We placed individuals for biomass analyses directly into vials with 70% ethyl alcohol during collection and while those for stable isotope and fatty acid composition analyses were placed directly into vials and frozen (at -20ºC for stable isotopes and -80ºC for fatty acid composition).

We identified insects and spiders to the family level. Arthropods were measured from the tip of head to the tip of abdomen using an ocular micrometer under a dissecting microscope at

6.3-25× magnification. Biomass, as dry mass, based on microscopy measurements was calculated using regression relationships of insect body length and dry mass from Sabo et al. (2002). We also measured dry mass directly by separating insects by order or family and then drying them for over 48 hours at ~50ºC, followed by weighing them on a Mettler Toledo AG245 balance (Mettler Toledo, LLC, Columbus, OH). We converted all dry biomass measurements into average dry biomass per day per area.

Avian sampling and foraging observations

We identified active Eastern Phoebe nests at stream sites in May 2015 and 2016 and monitored them from egg stage through hatching and, if initiated, through second clutch hatching. We sampled Eastern Phoebes under United States Fish and Wildlife Service migratory bird scientific collection permit #MB757670 and New York State Department of Environmental Conservation scientific collection permit #1477. All animal work was approved under Cornell Institutional Animal Care and Use Committee protocol #2001-0051. Chicks for stable isotope and fatty acid composition analyses were removed from nests by hand, placed into cotton geological sample bags, and kept warm with conspecifics before and after weighing and blood sampling. We weighed chicks two to five times per site with an Ohaus Scout Pro balance (Ohaus Corp., Parsipanny, NJ) and collected blood samples from each chick in the nest once in early development (before day five) and once in later development (after day nine). We took blood samples with 27G needles and collected a maximum of two non-heparinized capillary tubes of blood from each chick, which were immediately frozen.

We also quantified adult Eastern Phoebe foraging behavior at a subset of sites at the following streams: Carter Creek, Chaffee Creek, Michigan Hollow Creek, Cascadilla Creek,

Candor Creek, and Locke Creek between 10-June-2015 and 29-June-2015. We observed adults between 1200 and 1600 h at all sites and additionally between 0900 and 1000 h at Carter and Locke in order to increase the sample size of foraging attempts at those sites. A minimum of 14 foraging attempts were recorded at each site. At each site, 1-2 adults (parents) were observed foraging. We recorded all visible foraging attempts over the duration of the observation period and categorized them as foraging: directly from the stream, in the riparian zone, or in the terrestrial zone. We defined the riparian zone as the area within approximately 5 m of the stream bank, and the terrestrial zone as the area >5 m from the stream. We defined a foraging attempt as an instance in which a bird performed: a deliberate swoop or sally, a quick change in direction mid-flight, or leaving a perch, then swooping or sallying and quickly returning to the same or a different perch. We considered the following metrics of foraging preferences: 1) proportion of foraging attempts directly over the stream, 2) proportion of foraging attempts in the riparian area (≤ 5 m from the stream), 3) proportion of foraging attempts in the upland terrestrial area (> 5 m from the stream), and 4) proportion of foraging attempts in both the stream and riparian area.

Fatty acid composition analyses

We extracted fatty acids (FA) from whole insects and whole bird blood, derivatizing them to fatty acid methyl esters (FAMEs) with a modified one-step method, which is preferable for polyunsaturated FA (PUFA) extraction from low moisture samples (Garces and Mancha 1993, Zhou et al. 2008). We sampled the following insects for fatty acid composition analyses: freshwater Heptageniidae (Ephemeroptera), freshwater Perlidae (Plecoptera), terrestrial Coleoptera, terrestrial Diptera, terrestrial Hymenoptera, and terrestrial Lepidoptera. These taxa were all present in relatively high abundance and composed a large portion of biomass across

sites. We analyzed the fatty acid composition of whole blood from Eastern Phoebe chicks at a subset of sites that varied numerous environmental factors and spanned our land use gradient (Table 1): Miller Creek (n=3 chicks), West Candor Creek (n=5 chicks), and Locke Creek (n=3 chicks). All FA samples were analyzed from wet samples (previously frozen at -80ºC until extraction).

First, we added an aqueous reagent of methanol, 2,2-dimethoxypropane, and sulfuric acid and then an organic reagent of heptane and toluene to samples in test tubes. We vortexed and then shook samples in a water bath at 80°C for 2 h. After samples returned to room temperature, we added water saturated with NaCl to each sample, and vortexed and then centrifuged samples for 10 min at 3500 rpm. We transferred the top lipid layer to a clean test tube and added heptane to the initial tube followed by vortexing and another 10 min of centrifugation. We transferred the top lipid layer to new test tube, which was then dried down under N_2 gas. We transferred N_2-dried samples to stock vials in heptane and stored them at –80°C until quantification of FAMEs. We quantified FAMEs with the aid of a BPX-70 (SGE Inc., Ringwood, Victoria, Australia) column and a HP5890 series II GC-FID (Agilent Technologies, Santa Clara, California). We processed chromatogram data with PeakSimple 2.83 software (SRI Instruments, Torrance, California). We calculated response factors based on the reference standard 462a (Nu-Check Prep, Waterville, Minnesota). We identified FAMEs with the aid of a Varian (Agilent Technologies, Santa Clara California) Saturn 2000 ion trap with a Varian Star 3400 gas chromatography mass spectrometer run in chemical ionization mass spectrometry mode using acetonitrile as reagent gas and H_2 as a carrier gas (Van Pelt and Brenna 1999). We expressed FA composition data as percent of total FA.

Stable isotope and elemental analyses

We oven-dried all insect and bird blood samples for stable isotope analysis at ~50°C for >48 h. Freshwater insects used in stable isotope analyses included: Baetidae (Ephemeroptera), Chironomidae (Nematoceran Diptera), Heptageniidae (Ephemeroptera), Anisoptera, Perlidae (Plecoptera), Tipulidae (Nematoceran Diptera), and Trichoptera, and terrestrial insects included: Coleoptera, Diptera, Hymenoptera, and Lepidoptera. Samples for δ^2H analyses were equilibrated in Ithaca, New York, for ≥8 weeks prior to analysis. Samples for stable isotope and elemental analysis were ground and homogenized. We weighed ~0.5 mg of sample into Sn capsules for %C, %N, $\delta^{13}C$, and $\delta^{15}N$ analyses and ~0.3 mg of sample into Ag capsules for H and δ^2H analyses. Samples were analyzed at the Cornell Stable Isotope Laboratory in Ithaca, New York, on a Thermo Delta V Advantage isotope ratio mass spectrometer interfaced with a NC2500 elemental analyzer for $\delta^{13}C$ and $\delta^{15}N$ and with a temperature conversion elemental analyzer for δ^2H and Conflo III interface. $\delta^{13}C$, and $\delta^{15}N$ were standardized to methionine and an internal deer standard. δ^2H was standardized to Vienna Standard Mean Ocean Water based on internal laboratory standards including keratin and benzoic acid. We calculated insect carbon to nitrogen mass ratios (C:N) as %C divided by %N.

Elemental and fatty acid fluxes from arthropods

We estimated freshwater and terrestrial arthropod fluxes of C, N, ALA, and EPA to understand how the availability of freshwater and terrestrial nutrients changed throughout the Eastern Phoebe breeding season. To estimate fluxes of C and N, we multiplied arthropod dry mass per area per day by mean freshwater or terrestrial percent C and by mean freshwater or terrestrial percent N. To estimate fluxes of ALA and EPA, we multiplied arthropod dry mass per area per

day by percent lipid per dry mass by percent ALA and by percent EPA. We used percent lipid content for different arthropod taxa from Hanson et al. (1985) and Lease and Wolf (2011). We used mean values for lipid calculations for taxa for which we could not find published estimates of lipid content (Supplementary Table 1.1). In addition, we used mean freshwater percent ALA and percent EPA for freshwater insects that we captured but did not have direct measurements of fatty acid composition from and mean terrestrial percent ALA and percent EPA for terrestrial arthropods that we did not have direct measurements of fatty acid composition from (Supplementary Table 1.1). We used percent ALA and percent EPA values for Trichoptera (caddisflies) from a subset of sites (Twining et al. in prep; Supplementary Table 1.1). Note that our estimated fluxes of nutrients from freshwater insects and terrestrial arthropods are not directly comparable to one other because we used different trapping methods to collect freshwater and terrestrial arthropods.

Dietary analyses

We used the R package MixSIAR (Stock et al. 2016) to reconstruct Eastern Phoebe diets. Prior to running mixing models, we removed outlier high $\delta^{15}N$ terrestrial arthropod taxa that were unlikely to contribute substantially to Eastern Phoebe diets (*Drosophilia* spp., Staphylinidae, and Scarabidae) based on past diet studies (Kautza and Sullivan 2016) and their $\delta^{15}N$ values, which were far above those of Eastern Phoebes. We ran both $\delta^{15}N$ and $\delta^{13}C$ models and $\delta^{15}N$ and $\delta^{2}H$ models. We ran mixing models using trophic discrimination factors (TDF) for bulk $\delta^{13}C$ and $\delta^{15}N$ (2.71 +/- 0.38‰ for $\delta^{15}N$ and 0.29 +/- 0.12‰ for $\delta^{13}C$) that we developed based on comparisons of Tree Swallow chick blood samples relative to known food under experimental conditions (Twining and Shipley, in prep). These experimentally-estimated TDF values are

within range of those of other passerines (Healey et al. 2016). In $\delta^{15}N$ and $\delta^{2}H$ models, we assumed no $\delta^{2}H$ trophic discrimination. We used uniformed priors in all models (i.e., we started with assumption that Eastern Phoebes consume 50% freshwater insects and 50% terrestrial insects). All models included site as a random factor, canopy cover over the stream as a continuous factor, and were run with a long model run time to reach model convergence. See supplemental material for full MixSIAR diagnostic material.

Statistical analyses

To understand how nutritional composition and freshwater and terrestrial food availability varied across the landscape for Eastern Phoebes, we analyzed differences in percent ALA and percent EPA of freshwater insects and terrestrial arthropods by taxon, stream, and date by habitat origin (freshwater or terrestrial) using two sample t-tests. In addition, we used general linear models to analyze differences in ALA and EPA by insect taxon and by site. We also used general linear models to analyze within-taxon differences in ALA and EPA by site. We performed post-hoc Tukey tests to determine the directionality and significance of independent contrasts between insect taxon and sites. We did not analyze differences in percent DHA by either habitat origin or taxon because we only detected DHA in stoneflies. We also analyzed C:N of arthropods, as a proxy for lipid content, by habitat origin using two-sample t-tests and by taxon and by site using general linear models with post-hoc Tukey tests.

To understand how fluxes of biomass, ALA, and EPA from freshwater insects and terrestrial arthropods varied with sampling date, taxon, and stream, we analyzed flux estimate data with linear mixed effects models. Prior to running models, we log transformed all flux data. We included day of year and taxon as fixed effects and stream as well as taxon nested by stream

as random effects.

To understand how local factors including prey availability and quality as well as other environmental variables such as local land use influenced Eastern Phoebe diet, we related local factors to our mixing model results. Because $\delta^{15}N$ and $\delta^{13}C$ models and $\delta^{15}N$ and $\delta^{2}H$ models yielded similar estimates of Eastern Phoebe diet across the landscape and at individual sites (Supplementary Table 7), we related local factors only to the results of $\delta^{15}N$ and $\delta^{13}C$ models.

We also analyzed foraging preferences using general linear models as well as the following metrics of prey availability and quality: mean and maximum freshwater biomass flux, mean and maximum terrestrial biomass flux, mean freshwater and terrestrial ALA fluxes, mean freshwater and terrestrial EPA fluxes, mean freshwater and terrestrial percent ALA, mean freshwater and terrestrial percent EPA. We also considered the following metrics of riparian land use and land cover: percent agricultural land within 100 m radius of sampling site, percent agriculture within 25 m buffer zone of stream within 100 m upstream of sampling site, and canopy cover. Finally, we considered the following additional key metrics of local stream conditions in our general linear models: stream discharge, mean stream water temperature, mean chlorophyll *a* concentration (proxy for stream primary producer biomass), mean AFDM (proxy for stream microbial biomass), and total and soluble reactive phosphorus concentrations. All statistical analyses were conducted in R version 3.4.3.

Results

Environmental variability

The study sites we selected encompassed a wide range of local environmental conditions (Table

4.1). Dimensions one and two of correspondence analysis explained 40.6% and 21.7% of variability between sites respectively (Supplementary Figure 4.1). Dimensions one and two primarily captured variation in variables related to local land use and land cover, including stream canopy cover, local agricultural land use (agriculture within the 25 m buffer along a 100 m reach upstream of our sampling sites and agriculture within a 100 m radius of our sampling sites) as well stream primary producer biomass (measured as ash free dry mass and chlorophyll *a*), and the coefficient of variation in dissolved oxygen. Within these variables, canopy cover had strong negative correlations with percent agriculture within a 100 m radius of our sampling site, with stream temperature, and chlorophyll *a* whereas percent agriculture within a 100 m radius of our sampling site and stream temperature had strong positive correlations with chlorophyll *a* (Supplementary Figure 4.1). Chlorophyll *a* and ash free dry mass also had a high positive correlation, suggesting that stream primary producers had similar relationships with total epilithon biomass even across sites that differed substantially in local use and light levels (Supplementary Figure 4.2). Mean stream temperature, mean air temperature, the coefficients of variation in stream and air temperature, pH, conductivity, and mean dissolved oxygen concentrations captured a very limited amount of variability among sites (Supplementary Figure 4.1). Water-column inorganic nutrient concentrations (measured as TP and SRP) captured only a small amount of variance among sites (Supplementary Figure 4.1) and other than each other, they were not strongly correlated with environmental variables, including agricultural land use (Supplementary Figure 4.2), suggesting that agricultural land use effects on stream primary producer biomass primarily manifest themselves through effects on cover, not nutrient status.

Table 4.1 Stream study site descriptions. For canopy cover, air temperature, stream temperature, stream dissolved oxygen, chlorophyll *a*, ash free dry mass, total phosphorus, and soluble reactive phosphorus means and ± one standard error is shown.

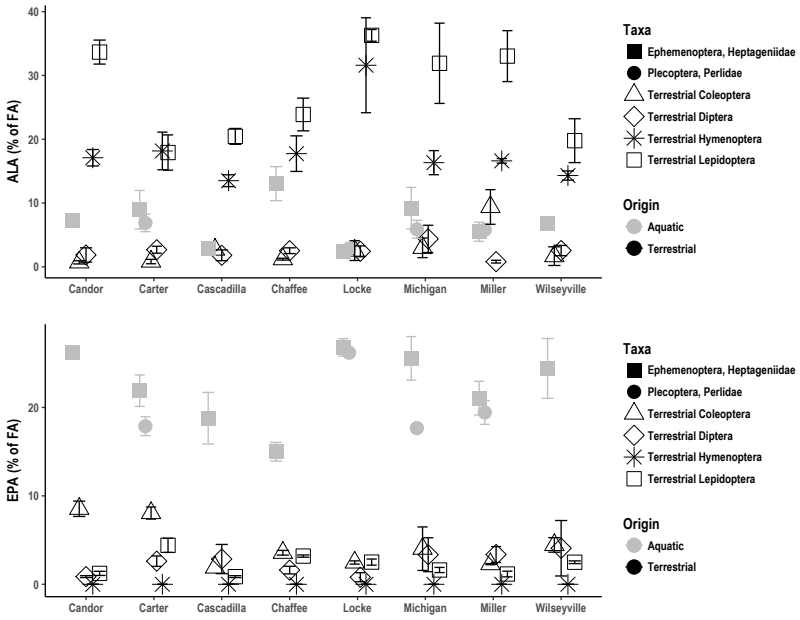
	Miller	Michigan Hollow	Wilseyville	Candor	Chaffee	Carter	Cascadilla	Locke
Latitude, Longitude (º)	42.29, -76.45	42.28, -76.49	42.29, -76.38	42.22, -76.41	42.31, -76.62	42.33, -76.66	42.44, -76.44	42.58, -76.53
Width (m)	2.5	5.5	4.5	13	1.5	2.5	4	6
Discharge (m^3/s)	0.48	0.96	1.06	2.30	0.03	0.65	0.36	0.76
Agricultural Land within 100 m radius (%)	0	42.4	63	38.1	0	0	62.6	25
Agricultural Land within 25 m buffer (%)	0	36.36	33.33	0	0	0	17.24	30.77
Canopy Cover (%)	79 ± 3	76 ± 7	0 ± 0	26 ± 3	73 ± 2	76 ± 9	19 ± 8	66 ± 8
Temperature (ºC)	15.26 ± 0.06	15.97 ± 0.06	17.19 ± 0.07	18.01 ± 0.05	13.76 ± 0.05	13.58 ± 0.05	17.79 ± 0.06	16.20 ± 0.06
Dissolved Oxygen (mg/L)	9.76 ± 0.02	9.52 ± 0.01	9.43 ± 0.02	8.54 ± 0.04	8.24 ± 0.02	8.36 ± 0.02	10.23 ± 0.01	9.36 ± 0.01
pH	8.2	7.7	7.68	7.86	7.81	8.6	7.96	8.12
Conductivity (mS/cm)	124	64	211	259	130	55	364	589
Chlorophyll *a* (mg/cm^2)	0.84 ± 0.38	0.41 ± 0.17	3.14 ± 0.36	2.60 ± 0.42	0.64 ± 0.19	0.23 ± 0.09	7.80 ± 1.88	1.05 ± 0.30
Ash Free Dry Mass (mg/cm^2)	0.62 ± 0.09	0.58 ± 0.02	0.51 ± 0.24	2.61 ± 0.67	0.42 ± 0.06	0.56 ± 0.10	4.20 ± 1.05	1.11 ± 0.42
Total Phosphorus (µg/L)	17.45 ± 1.50	88.82 ± 6.61	35.42 ± 9.25	61.08 ± 10.44	158.63 ± 53.00	33.6 ± 9.03	73.85 ± 17.39	108.75 ± 27.14
Soluble Reactive Phosphorus (µg/L)	3.91 ± 0.45	12.82 ± 3.10	7.55 ± 0.50	9.31 ± 2.51	18.30 ± 3.26	10.16 ± 2.09	6.77 ± 0.28	16.80 ± 2.05

Nutritional composition

We expected terrestrial and freshwater insects to contain similar amounts of the HUFA precursor ALA, but found that percent ALA was two-fold higher in terrestrial insects than in freshwater

insects (t = -4.618, df = 115.68, p < 0.0001; Figure 4.1), driven mainly by terrestrial bees and butterflies, which had significantly higher percent ALA than either stoneflies or mayflies, while terrestrial flies and beetles had significantly lower percent ALA than either pollinators or freshwater insects (Table 4.2). We also hypothesized that freshwater insects across the landscape would be higher in HUFAs, especially EPA, than would terrestrial insects. Percent EPA was almost ten-fold higher in freshwater insects than in terrestrial insects (t = 23.161, df = 40.747, p < 0.0001; Figure 4.1). While stoneflies and mayflies both had significantly higher percent EPA than any terrestrial insects, percent EPA was also significantly higher in terrestrial flies and beetles than in terrestrial butterflies or bees, which contained no detectable EPA (Table 4.2). Only stoneflies contained DHA within range of our detection limit. Across taxa, percent ALA and EPA did not differ by site (Supplementary Table 4.2), while within taxa, percent ALA and EPA differed inconsistently among sites (Supplementary Table 4.3).

Figure 4.1 Mean and standard error of percent (a) ALA and (b) EPA of insects across sites.



Freshwater insects had significantly higher C:N than terrestrial insects (mean freshwater C:N = 4.825 versus mean terrestrial C:N = 4.558; $t = 2.618$, $df = 168.38$, $p < 0.01$). Mayflies had higher C:N than stoneflies or bees (Table 4.2) while dobsonflies (Megaloptera) had the highest C:N (Table 4.2). Across taxa, C:N did not differ by site (Supplementary Table 4.2). Eastern Phoebe chick blood samples were composed of an average of 9.03% ALA, 1.61% docosapentanoic acid (DPA), and 3.78% DHA (Supplementary Table 4.4). We found only trace amounts of EPA in Eastern Phoebe blood samples.

Table 4.2 General linear models of percent ALA, percent EPA, and C:N by insect taxa. SE is standard error, LSM is least squares means, and df is degrees of freedom.

ALA (percent of fatty acid composition)

	SE	LSM	t-value	p-value	Direction
Intercept	1.122	---	6.169	< 0.0001	---
A. Stoneflies	1.972	5.305	-0.818	0.415	T. Beetles, T. Flies < A. Stoneflies, A. Mayflies < T. Bees < T. Butterflies
A. Mayflies	---	6.919	---	---	
T. Beetles	1.645	2.476	-2.702	< 0.01	
T. Flies	1.586	2.371	-2.867	< 0.005	
T. Bees	1.604	18.325	7.111	< 0.0001	
T. Butterflies	1.570	27.116	12.868	< 0.0001	
Null Deviance: 14700.8 on 122 df			Residual Deviance: 3385.1 on 117 df		

EPA (percent of fatty acid composition)

	SE	LSM	t-value	p-value	Direction
Intercept	0.615	---	36.32	< 0.0001	---
A. Stoneflies	1.081	20.531	-1.66	< 0.10	T. Bees, T. Butterflies < T. Flies, T. Beetles < A. Stoneflies, A. Mayflies
A. Mayflies	---	22.325	---	---	
T. Beetles	0.901	4.766	-19.48	< 0.0001	
T. Flies	0.860	2.442	-23.12	< 0.0001	
T. Bees	0.879	0	-25.40	< 0.0001	
T. Butterflies	0.860	2.185	-23.41	< 0.0001	
Null Deviance: 10625.1 on 123 df			Residual Deviance: 1025.3 on 118 df		

C:N

	SE	LSM	t-value	p-value	Direction
Intercept	0.24673	---	17.703	< 0.0001	---
A. Stoneflies	0.28215	4.406	0.134	0.894	A. Stoneflies, T. Bees < A. Mayflies
A. Mayflies	0.26358	5.046	2.573	< 0.05	

A. Caddisflies	0.26956	4.817	1.668	0.0964
A. Dobsonflies	0.55172	5.468	1.994	< 0.05
A. Odonates	0.30930	4.723	1.149	0.252
A. Flies	0.28699	4.772	1.407	0.160
T. Beetles	0.30219	4.703	1.109	0.268
T. Flies	---	4.368	---	---
T. Bees	0.30219	4.464	0.319	0.750
T. Butterflies	0.30017	4.600	0.774	0.439
Null Deviance: 251.29 on 328 df			Residual Deviance: 233.04 on 319 df	

Arthropod fluxes

We expected both emergent freshwater insect and terrestrial fluxes to be highly variable across sites and among dates. We found that fluxes of biomass, ALA, and EPA from freshwater insects differed significantly by day of year (Figure 4.2; Supplementary Table 4.5): freshwater biomass was significantly greater across the landscape on day 150 (May 30). For biomass fluxes, within stream variation (σ^2) was much greater than among stream by taxon and among stream variation (τ and ICC). For ALA and EPA fluxes, both within and among stream variation were very low and had little clear signal in our models (Supplementary Table 4.5). Although different freshwater insect orders and sub-orders dominated at individual sites, fluxes of biomass, ALA, and EPA from freshwater insect did not differ significantly among taxa (Figure 4.2; Supplementary Table 4.5). Across the landscape, no single freshwater insect taxon or stream dominated biomass or nutrient fluxes during our sampling period (Figure 4.2).

Fluxes of biomass, ALA, and EPA from terrestrial arthropods, like those from freshwater insects, differed significantly by day of year and were also highest on day 150 (Figure 4.3; Supplementary Table 4.5). As was the case for freshwater biomass, variation in terrestrial biomass fluxes was greater within streams than among streams (Figure 4.3; Supplementary Table 4.5). Like freshwater ALA and EPA fluxes, within and among stream variation in terrestrial

ALA and EPA fluxes were both very low and had little clear signal in our models (Supplementary Table 4.5). Unlike fluxes of freshwater biomass, ALA, or EPA, terrestrial fluxes did differ significantly by taxon (Supplementary Table 4.5). In particular, Coleoptera had significant associations with increased biomass fluxes and with EPA fluxes (Figure 4.3; Supplementary Table 4.5).

Throughout the Eastern Phoebe breeding season, fluxes of EPA from freshwater insects were significantly greater than fluxes of ALA from freshwater insects (Figure 4.3; $t = -6.56$, $df = 450$, $p < 0.0001$). In contrast, fluxes of ALA from terrestrial arthropods were significantly greater than fluxes of EPA from terrestrial arthropods (Figure 4.3; $t = 3.84$, $df = 470$, $p = < 0.001$).

Figure 4.2 Mean (a) freshwater insect biomass flux by taxon and stream, (b) freshwater insect ALA flux by taxon and stream, and (c) freshwater insect EPA flux by taxon and stream

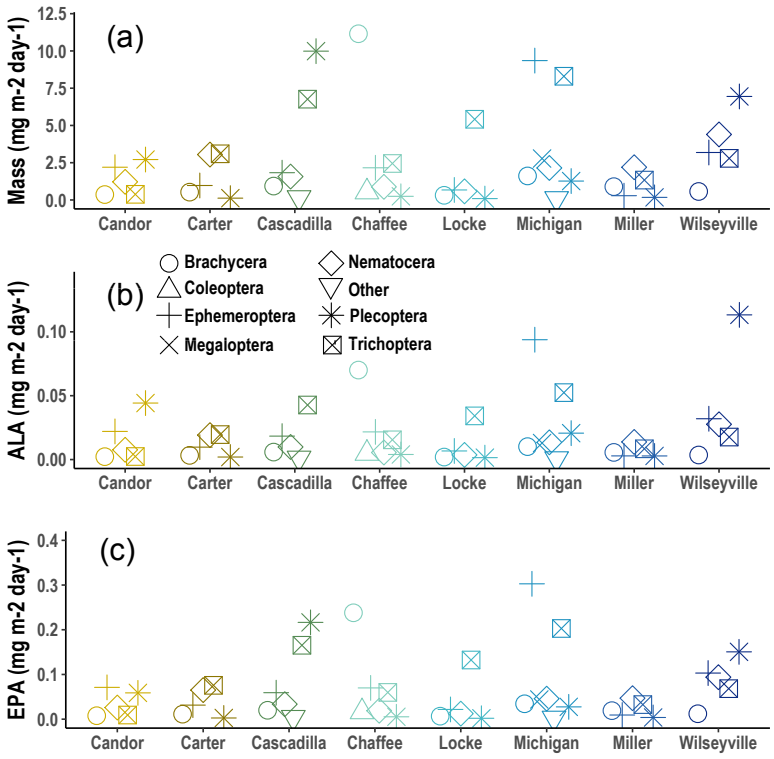
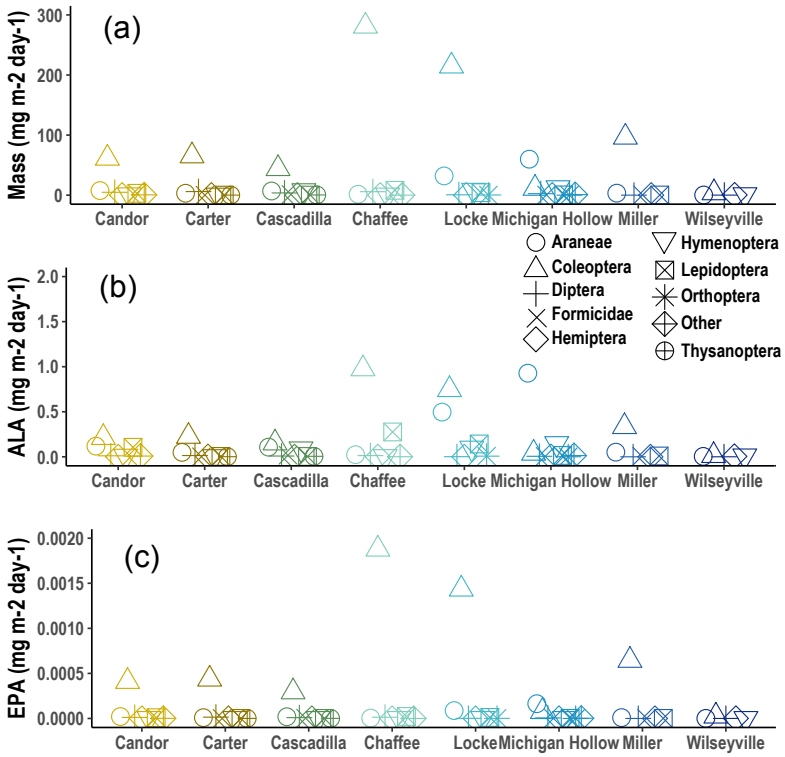


Figure 4.3 Mean (a) terrestrial arthropod biomass flux by taxon and stream, (b) terrestrial arthropod ALA flux by taxon and stream, (c) terrestrial arthropod EPA flux by taxon and stream

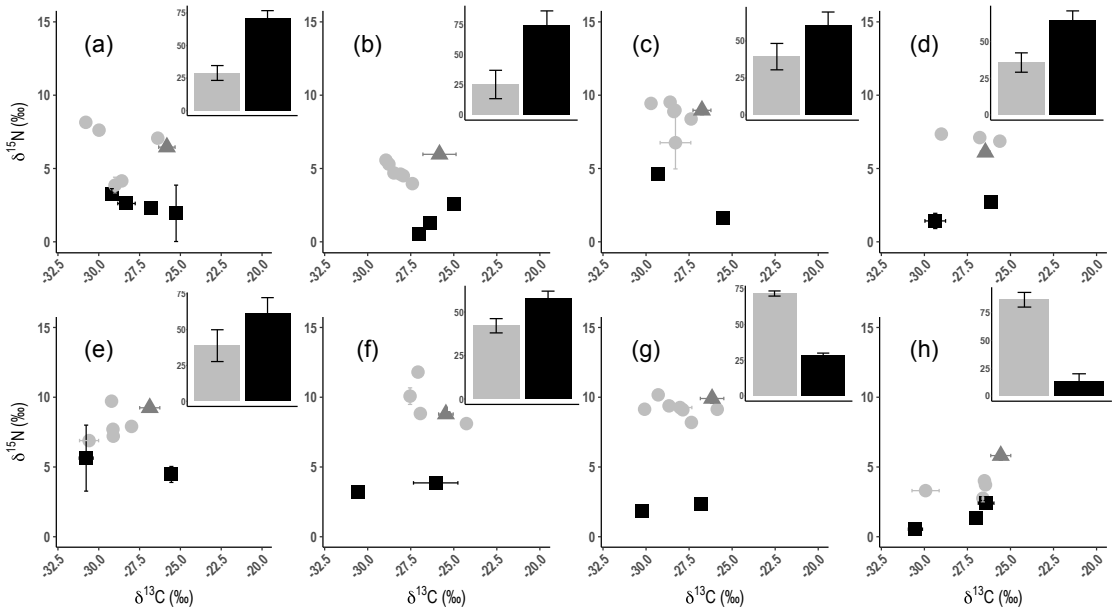


Eastern Phoebe foraging and consumption

Based on our hypothesis that freshwater resources would be higher quality than terrestrial resources and because nests were under bridges directly above streams at the majority of our sites, we expected Eastern Phoebes to spend the majority of their time foraging over streams or within the riparian area. Behavioral observations revealed that Eastern Phoebe adults not only foraged directly over streams and in the riparian area, but also in upland terrestrial habitats. However, Eastern Phoebes tended to forage in the terrestrial habitat within approximately 50-60 m from the stream banks, often much closer. The proportion of total foraging attempts made over the stream decreased with increasing percent canopy cover over the stream (linear regression, N = 6 sites, f-value = 16.264, df = 5, R^2 = 0.803, p-value < 0.05).

Across the landscape, all Eastern Phoebe chicks consumed a mix of both freshwater and terrestrial prey (Figure 4.4). $\delta^{15}N$ and $\delta^{13}C$ models and $\delta^{15}N$ and $\delta^{2}H$ models yielded similar estimates of Eastern Phoebe chick diet across the landscape (47.7% freshwater and 52.3% terrestrial for $\delta^{15}N$ and $\delta^{13}C$ models and 45.7% freshwater and 53.4% terrestrial for $\delta^{15}N$ and $\delta^{2}H$ models) and at individual sites (Supplementary Table 4.6). Eastern Phoebes consumed far more freshwater prey at Carter and Candor (71-86% freshwater prey) than other sites (mean of 35% freshwater prey) whereas Eastern Phoebes consumed the most terrestrial prey at Miller (Figure 4.4; Supplementary Table 4.6). We found significant positive relationships between the strength of freshwater subsidies and mean terrestrial insect percent EPA ($R^2 = 0.48$) and stream discharge ($R^2 = 0.14$; Supplementary Table 4.7). We found significant negative relationships between the strength of freshwater subsidies and mean stream dissolved oxygen concentrations ($R^2 = 0.42$), mean freshwater biomass fluxes ($R^2 = 0.33$), mean freshwater EPA fluxes ($R^2 = 0.32$), agricultural land use within 25 m buffer zone ($R^2 = 0.32$), mean freshwater insect ALA ($R^2 = 0.27$), riparian foraging ($R^2 = 0.27$), maximum ($R^2 = 0.17$) freshwater insect biomass fluxes, mean terrestrial ALA fluxes ($R^2 = 0.12$), and mean terrestrial percent ALA ($R^2 = 0.12$; Supplementary Table 4.7).

Figure 4.4 Mean carbon and nitrogen stable isotope values of aquatic insects (circles), terrestrial insects (squares), and Eastern Phoebe chick blood (triangles) across sites. Main plot Eastern Phoebe data points do not include displacement due to trophic discrimination. Error bars represent standard deviation. Subplots show mean and standard deviation of mixing model-generated percent aquatic (gray) and terrestrial (black) prey contributions to Eastern Phoebe diets across sites. Sites are: (a) Miller Creek, (b) Michigan Hollow Creek, (c) Wilseyville Creek, (d) Chaffee Creek, (e) Cascadilla Creek, (f) Locke Creek, (g) West Candor Creek, and (h) Carter Creek.



Discussion

Although riparian predators like insectivorous birds live in terrestrial habitats, they may rely on freshwater subsidies to provide them with locally scarce nutritional resources (e.g., Dodson et al. 2016; Twining et al. 2017). We found that freshwater subsidies were a source of terrestrially-scarce resources for riparian insectivores: across a diverse agricultural and forested landscape, freshwater insects were significantly richer in HUFAs, specifically EPA, than were terrestrial insects (Table 4.2; Figure 4.1), following our expectations. Terrestrial insects had significantly higher percentages of the HUFA precursor ALA than did freshwater insects (Table 4.2; Figure 4.1). We also hypothesized that the strength of freshwater subsidies to riparian predators would either be related to: 1) local factors including the relative availability and quality of prey impact,

or 2) species-level nutritional requirements that did not vary locally. While at least 25% of diet in Eastern Phoebes, our representative riparian avian predator, was comprised of freshwater insects across the landscape, among sites Eastern Phoebe chicks had substantially different percentages of freshwater insects in their diets (Figure 4.4). However, we found little evidence to support our hypothesis that local factors, including freshwater or terrestrial prey availability (flux sizes) or quality (fatty acid composition) as well as environmental factors, were strongly associated with the strength of freshwater insect subsidies to Eastern Phoebes (Supplementary Table 4.7).

In light of the stark differences in fatty acid composition at the base of freshwater and terrestrial food webs and the differences we found between freshwater and terrestrial insects, we hypothesized that Eastern Phoebes might engage in nutrient-specific foraging (e.g., Mayntz et al. 2005) for HUFA from freshwater sources across the landscape regardless of local differences in prey availability or quality. Birds have specific appetites for minerals including calcium (Hughes and Wood-Gush 1971a; Reynolds and Perrins 2010) and sodium (Hughes and Wood-Gush 1971b) as well as organic compounds such as thiamine (Hughes and Wood-Gush 1971b) and carotenoids (Senar et al. 2010). In natural systems, specific appetites can lead birds to consume resources that are not part of their ordinary diets during specific life stages (Reynolds and Perrins 2010). For example, MacLean (1974) found lemming bones in the stomachs of female Sandpipers (*Calidris spp*.) during egg laying. Some studies suggest that shorebirds may engage nutrient-specific foraging for HUFAs at migratory stop-over points (Maillet and Weber 2006; Maillet and Weber 2007). Intriguingly, we found that Eastern Phoebe chicks consume a minimum of 25% of their diet as HUFA-rich freshwater insects at all of our sites. However, without additional data on Eastern Phoebe preferences for HUFA-rich versus HUFA-poor foods or data on the ability of birds to detect the HUFA content of individual flying insects, we cannot

make reliable inferences about nutrient-specific foraging for HUFAs. In light of the importance of HUFAs for Eastern Phoebes and other aerial insectivores, experimental studies quantifying specific appetites for HUFAs are vitally needed.

Foraging adult Eastern Phoebes in natural ecosystems are likely considering more than just the nutritional content of prey. Consumers in the wild must satisfy their nutritional needs while also considering numerous other factors such as predation when making foraging decisions (e.g., Krebs 1980; Whittingham and Evans 2004). Therefore, we also hypothesized that adult foraging behavior and its relationship with environmental variables might influence freshwater subsidies to chicks. At the six sites where we performed foraging observations, we found that adult Eastern Phoebes were more likely to forage directly over the stream at sites with less riparian canopy cover and chicks of parents who made more foraging attempts within the riparian zone consumed more freshwater insects (Supplementary Table 4.7). However, across all sites we found that canopy cover itself (Supplementary Table 4.7) was not a significant predictor of the strength of freshwater subsidies to Eastern Phoebe chicks.

Previous studies on freshwater insect subsidies to riparian birds have generally emphasized patterns generated by subsidy size. For example, in their landmark study on reciprocal subsidies between stream and riparian food webs, Nakano and Murakami (2001) focused on seasonal changes in the relative availability of freshwater insects and terrestrial arthropods. They found that seasonal shifts in resource availability explained seasonal shifts in diet for riparian birds (Nakano and Murakami 2001). Uesugi and Murakami (2007) also found that seasonal patterns in freshwater and terrestrial insect biomass explained patterns in bird distributions. We concentrated on freshwater subsidies to chicks in the late spring during the Eastern Phoebe breeding season because HUFA needs in avian insectivores are likely to be high

during this period of rapid growth and development.

If differences in local food availability or the availability of high quality food predict the strength of freshwater subsidies to riparian consumers, then we would expect to find differences in diet composition based on the relative availability of freshwater or terrestrial insect biomass and fatty acid fluxes. Although our sites varied in numerous environmental characteristics (Table 4.1; Supplementary Figure 4.1-4.2), we found few consistent differences in either mean freshwater insect fluxes or mean terrestrial arthropod fluxes across sites (Supplementary Table 4.7; Figure 4.2-4.3). One logistical limitation that we faced was that we were only able deploy three traps of each type across our eight sites. Thus, we may have either under- or over-estimated flux sizes on given dates due to spatial patchiness within sites. However, across our sampling period we found that temporal variation in both freshwater and terrestrial fluxes within sites was much greater than spatial variation in fluxes across the landscape or between traps (Supplementary Table 4.5). In light of the high temporal variability that we found, it is not surprising that we found only weak and negative associations between percent freshwater diet across streams and either freshwater biomass fluxes or freshwater omega-3 fatty acid fluxes (Supplementary Table 4.7). While the weak, but positive association that we found between percent freshwater diet and stream discharge (Supplementary Table 4.7) suggests that adult Eastern Phoebes foraging along larger streams may feed chicks more freshwater insects, the negative association that we found between percent freshwater diet and riparian foraging attempts suggests that relationships between short-term foraging observations and diet are far from straightforward (Supplementary Table 4.7).

If freshwater subsidies to Eastern Phoebe chicks across all of our sites allowed all chicks to meet their minimum HUFA needs, freshwater prey availability and quality did not have had

the landscape-level effects that we expected them to have. In past laboratory studies, we showed that chicks grew faster and were in better condition when fed a diet with 7.18% of fatty acids as total HUFAs (3.74% as EPA and 3.44% as DHA; Twining et al. 2016b) than when fed a diet with 2.89% of fatty acids as total HUFAs (1.47% as EPA and 1.42% as DHA; Twining et al. 2016b). Here, we found that freshwater insects contained approximately 21.75% of fatty acids as EPA whereas terrestrial insects contained approximately 2.29% of fatty acids as EPA (Supplementary Table 4.1). This means that a diet of terrestrial insects alone is likely to limit developmental performance because terrestrial insects do even not supply chicks with the total HUFA levels found in our low performing treatments. In order to maintain a diet with at least 7.18% of fatty acids as HUFAs, Eastern Phoebe chicks must consume at least 25% of their diet as freshwater insects. Across our eight sites, Eastern Phoebe chicks consumed a minimum of 25% of diet as freshwater insects (Supplementary Table 4.6). We are admittedly somewhat limited in our ability to make comparisons between EPA and total HUFAs (EPA and DHA) in our laboratory experiments. However, the correspondence between Eastern Phoebe minimum HUFA requirements and freshwater insect consumption suggests that all of Eastern Phoebe chicks that we sampled may have received sufficient dietary HUFAs, in terms of EPA, from emergent freshwater insect subsidies.

Like previous studies (e.g., Popova et al. 2017; Martin-Creuzburg et al. 2017), we only found DHA in predatory insects (Perlidae stoneflies) and only in low percentages. DHA is appears to be scarce or absent in many emergent freshwater insects (e.g., Hixson et al. 2015; Guo et al. 2016s). In contrast, we found that Eastern Phoebe blood, which did contain DHA, contained only trace amounts of EPA (Supplementary Table 4.4). This suggests that chicks must have gotten their DHA either from ALA or EPA in diet. ALA conversion to DHA typically

requires seven different elongation and desaturation steps while EPA conversion to DHA usually requires only four of those steps (Hixson et al. 2015). In an experimental isotopic dosing, we found low ALA to EPA and low ALA to DHA conversion efficiency in other riparian insectivore chicks (Twining et al. 2018). In addition, although we found DPA, the intermediate between EPA and DHA, in chick blood we did not find any other intermediates between ALA and DHA (Supplementary Table 4.4). Thus, we argue that EPA, primarily from freshwater insects, conversion to DHA in Eastern Phoebes is more likely than ALA to DHA conversion.

Preferential retention of high quality nutritional resources like HUFAs may also help explain why we did not see strong effects of subsidy size and the relative availability of HUFA-rich freshwater insects on riparian insectivore chicks across sites. Animals (Hessen and Leu 2006), including other riparian aerial insectivores (Twining et al. 2016b), can preferentially retain physiologically vital fats like HUFAs, while preferentially oxidizing other fats for fuel. Compound-specific stable isotope analyses of Eastern Phoebe chick diets show that regardless of the contribution of freshwater insects to overall diets, chicks obtain their EPA from HUFA-rich freshwater sources while obtaining less physiologically-vital fats, like saturated fats, from both freshwater and terrestrial sources (Twining 2018). HUFA retention may be especially important during the rapid chick growth phase for altricial species because their parents invest little ALA or HUFAs in eggs (Speake and Wood 2005). Eastern Phoebe chicks and other temperate altricial aerial insectivore chicks nearly double in biomass every other day and reach mature size within approximately three weeks (Murphy 1981; Zach and Mayoh 1982). Dietary HUFAs during the rapid growth period increase chick body condition and growth rate in both Eastern Phoebes (Twining 2018) and Tree Swallows (Twining et al. 2016b). During this period, it is likely crucial for chicks to retain, convert, and deposit physiologically important compounds like HUFAs into

tissue to use throughout their lifetime. To fuel their rapid growth, chicks may preferentially use less physiologically-vital fatty acids and other compounds from either freshwater or terrestrial systems as fuel. Therefore, as long as chicks have access to enough freshwater prey to meet physiological requirements, they may rely on either freshwater or terrestrial prey for energy.

Numerous studies have documented the importance of freshwater-derived energy in terrestrial food webs (Muehlbauer et al. 2014). In their meta-analysis, Muehlbauer et al. (2014) found that 50% stream subsidies are concentrated within 1.5m of the banks and only about 10% of subsidies make it beyond 500 m, suggesting that subsidies have the strongest ecological impacts right along the ecosystem boundaries. Our research suggests that ecologists must consider subsidies for consumers in terms of quality as well as quantity. We argue that the 50% of subsidies that do make it more than 1.5 m away from streams may have a much wider influence on a diversity of terrestrial consumers than previously appreciated. For example, Popova et al. (2017) found that Odonates, which are particularly strong fliers, dispersed substantial quantities of EPA and DHA up to 4 km from a lakeshore. The effects of small, high quality subsidies are likely to be especially relevant for highly mobile foragers like birds or bats. Just as birds are known to forage well beyond their nests to obtain calcium for egg laying (Reynolds and Perrins 2010), a few foraging short trips within a riparian area or a few freshwater insects within each insect bolus for chicks with may similarly allow aerial insectivores to satisfy their chicks' HUFA needs.

Ultimately, the importance of ecosystem subsidies depends upon relative differences in resource availability between donor and recipient ecosystems as well as the degree to which local factors within both the donor and recipient system allow resource movement to occur (Polis et al. 1997). We considered whether nutritional needs or local factors predicted the strength of

freshwater subsidies to Eastern Phoebe chicks. We found little evidence for our hypothesis that local factors including subsidized prey availability dictated the overall degree of freshwater subsidies in a complex natural system. Instead, we found that predators relied upon high quality subsidized resources across the landscape. While past studies have overwhelmingly focused on resource availability as a driver of subsidy strength, our results show that when there are major differences in food quality for consumers between local resources and subsidies, subsidy size itself may be less important than subsidy quality.

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SUPPLEMENTARY MATERIALS – CHAPTER FOUR

Figure S4.1 Correspondence analysis between environmental variables across sites. Gray circles represent study sites and black triangles represent variables.

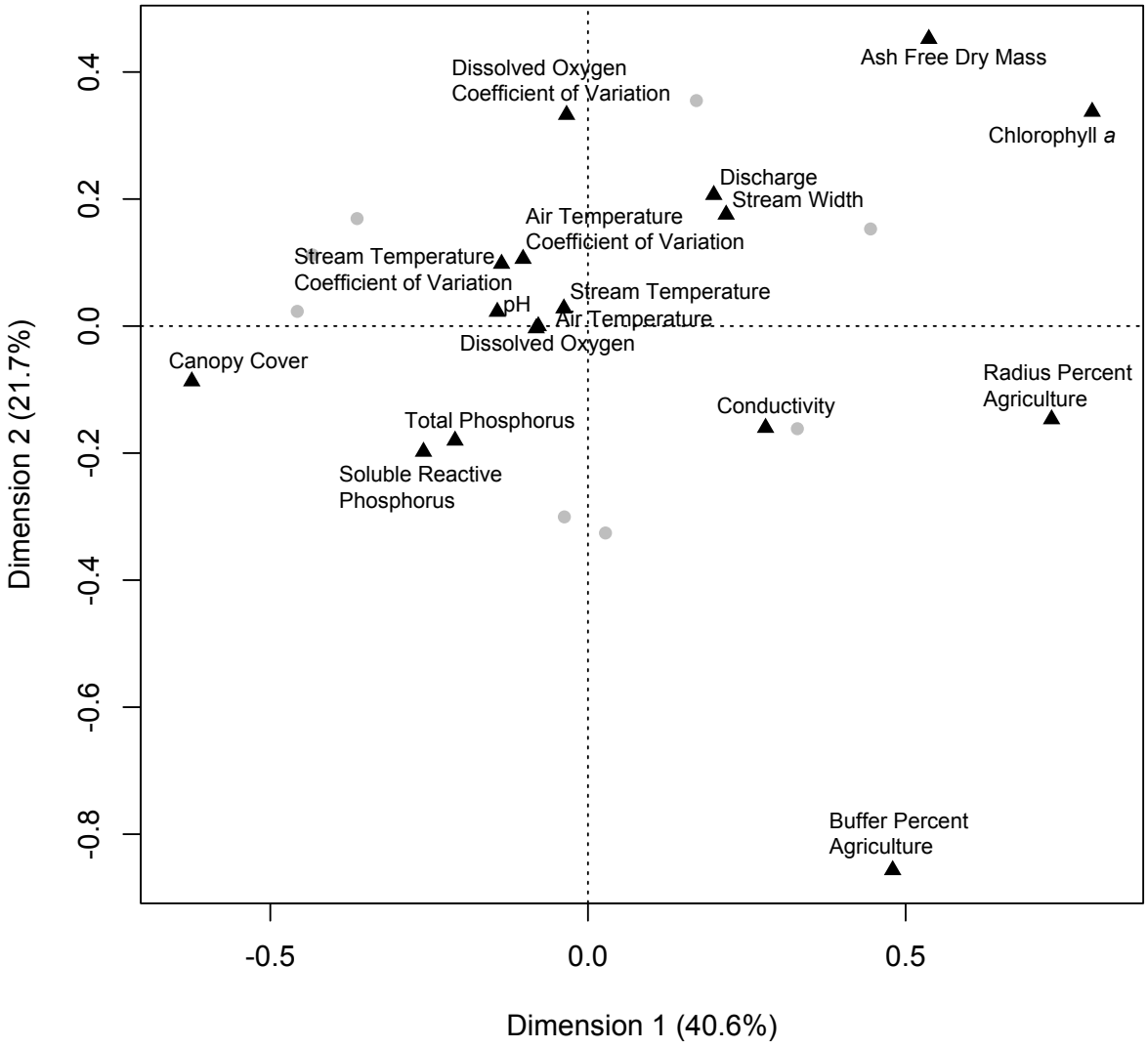


Figure S4.2 Pearson's correlation coefficients between environmental variables

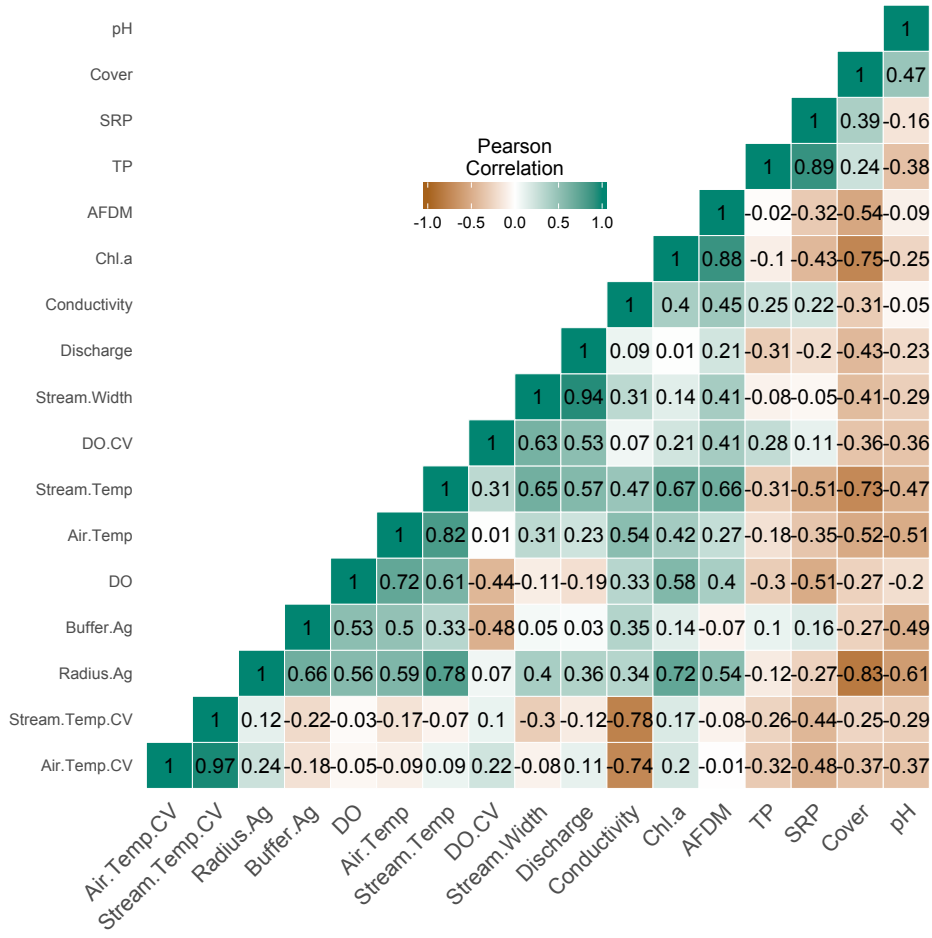


Figure S4.3 Posterior density plot for Miller Creek for $\delta^{15}N$ and $\delta^{13}C$ model

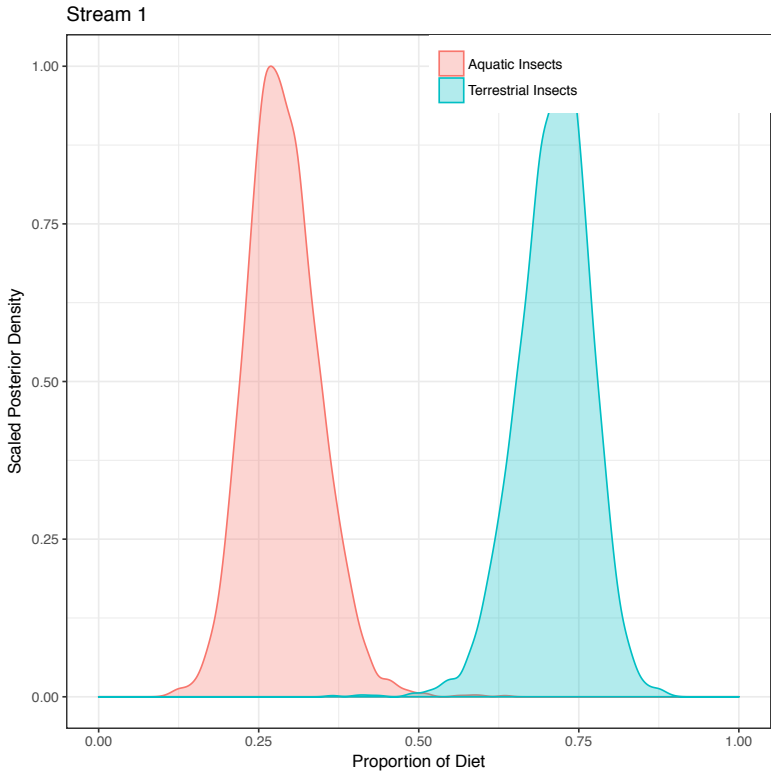


Figure S4.4 Posterior density plot for Michigan Hollow Creek for $\delta^{15}N$ and $\delta^{13}C$ model

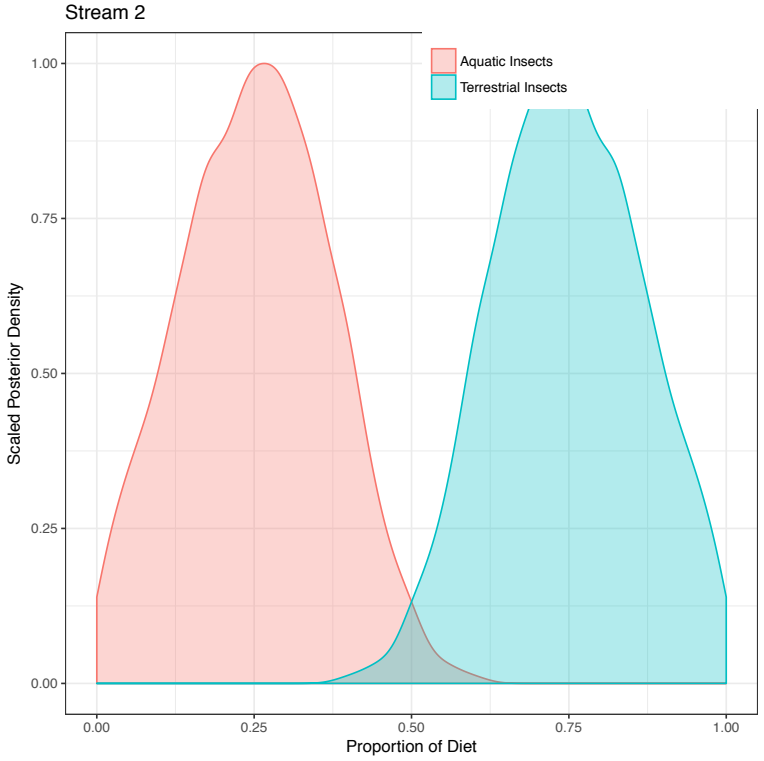


Figure S4.5 Posterior density plot for Wilseyville Creek for $\delta^{15}N$ and $\delta^{13}C$ model

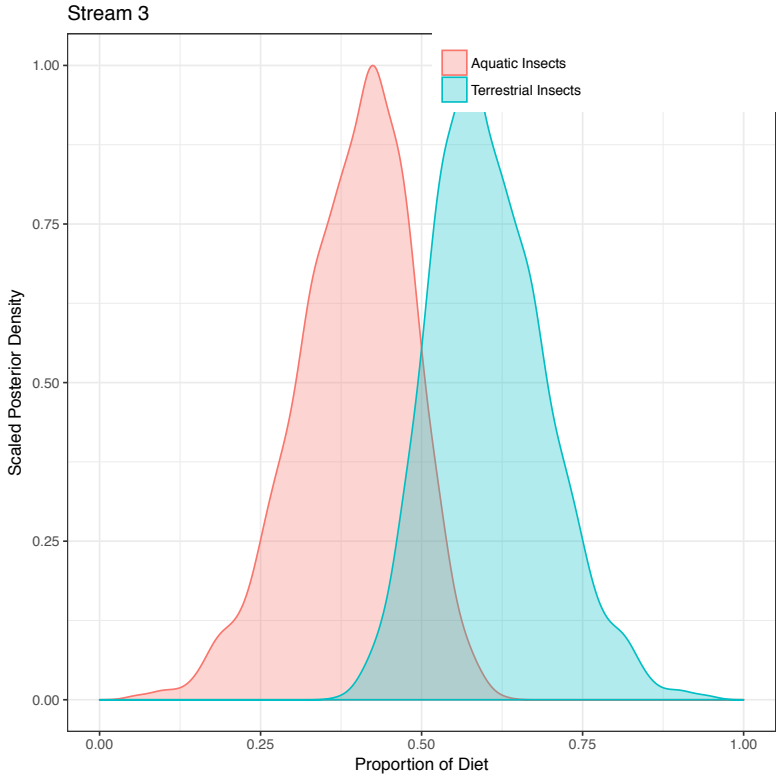


Figure S4.6 Posterior density plot for West Candor Creek for $\delta^{15}N$ and $\delta^{13}C$ model

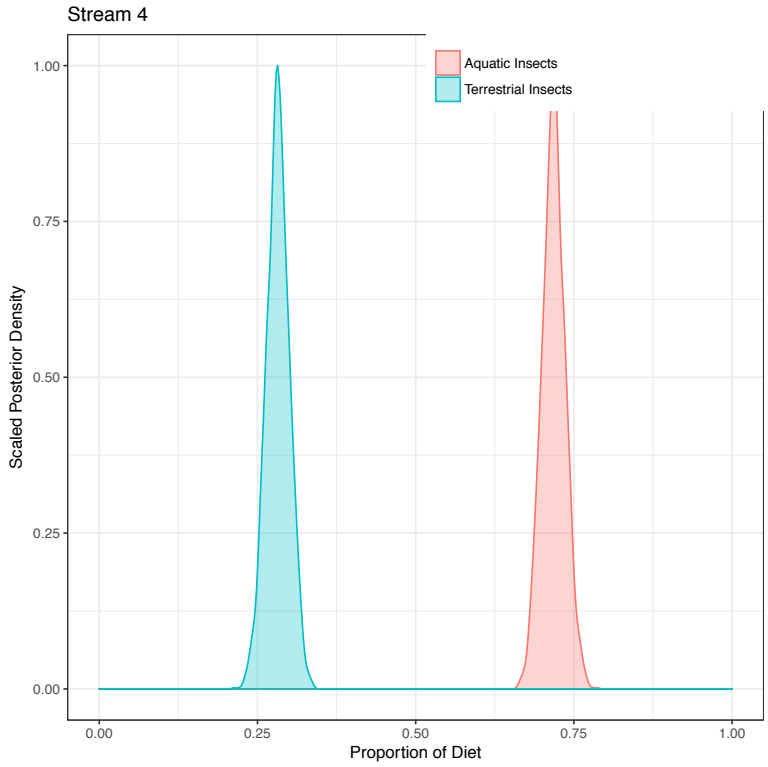


Figure S4.7 Posterior density plot for Chaffee Creek for $\delta^{15}N$ and $\delta^{13}C$ model

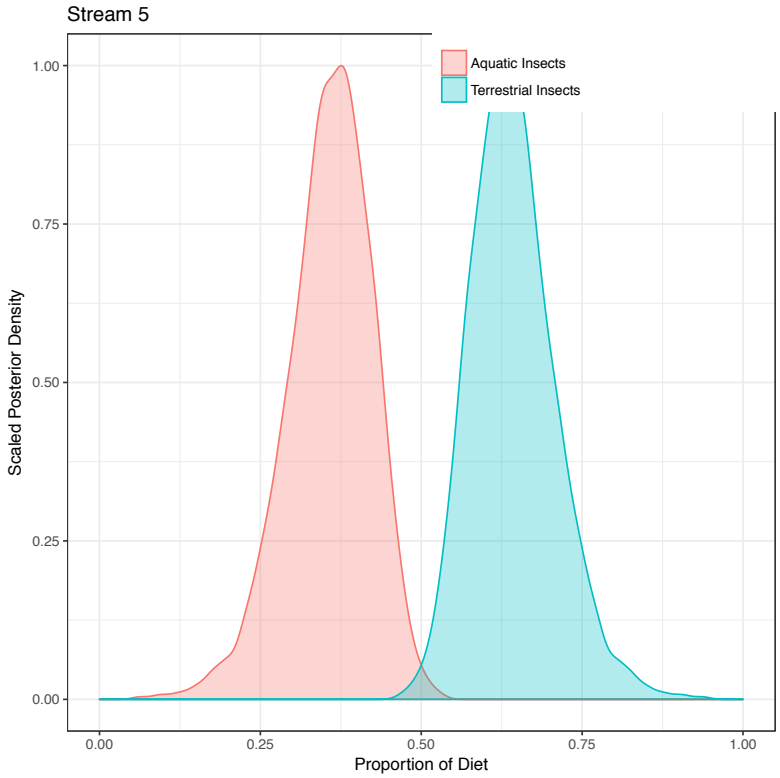


Figure S4.8 Posterior density plot for Carter Creek for $\delta^{15}N$ and $\delta^{13}C$ model

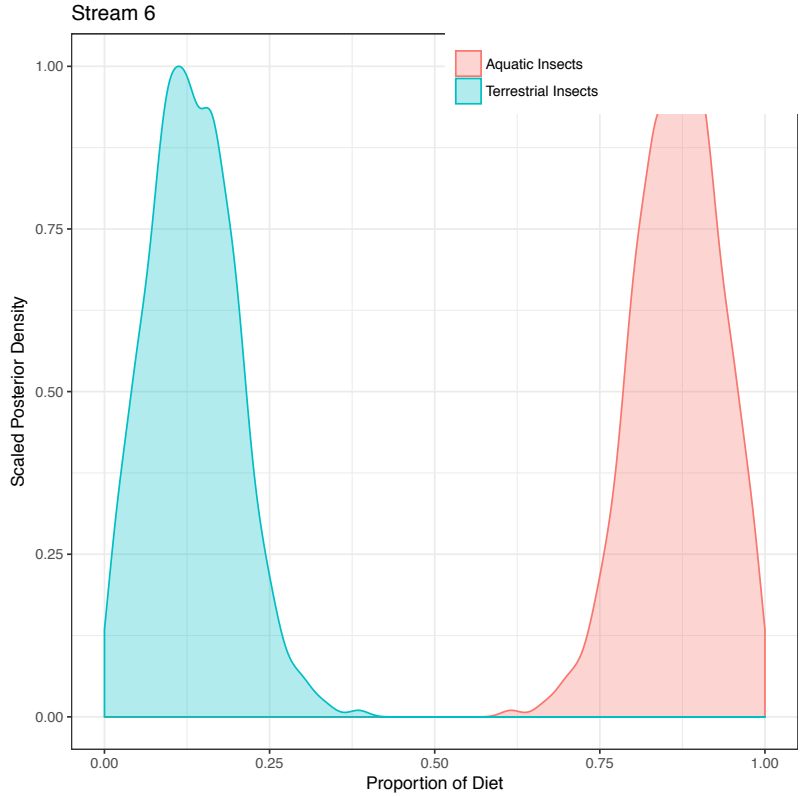


Figure S4.9 Posterior density plot for Cascadilla Creek for $\delta^{15}N$ and $\delta^{13}C$ model

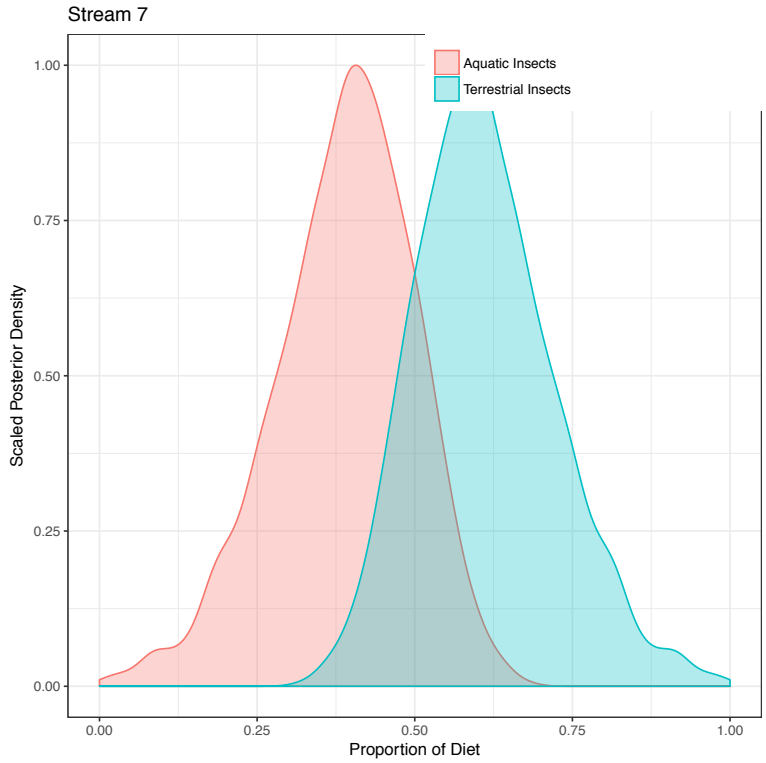


Figure S4.10 Posterior density plot for Locke Creek for $\delta^{15}N$ and $\delta^{13}C$ model

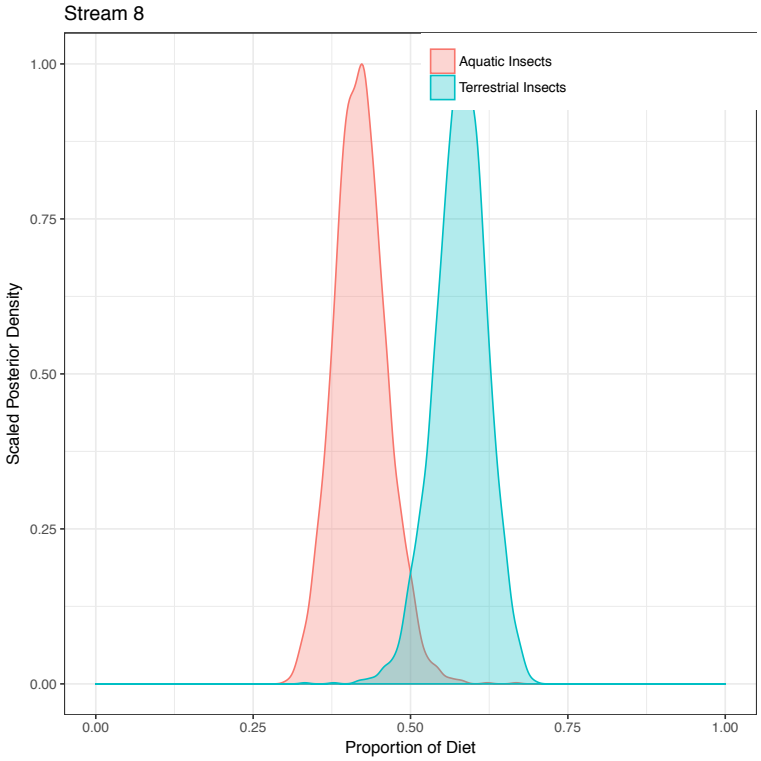
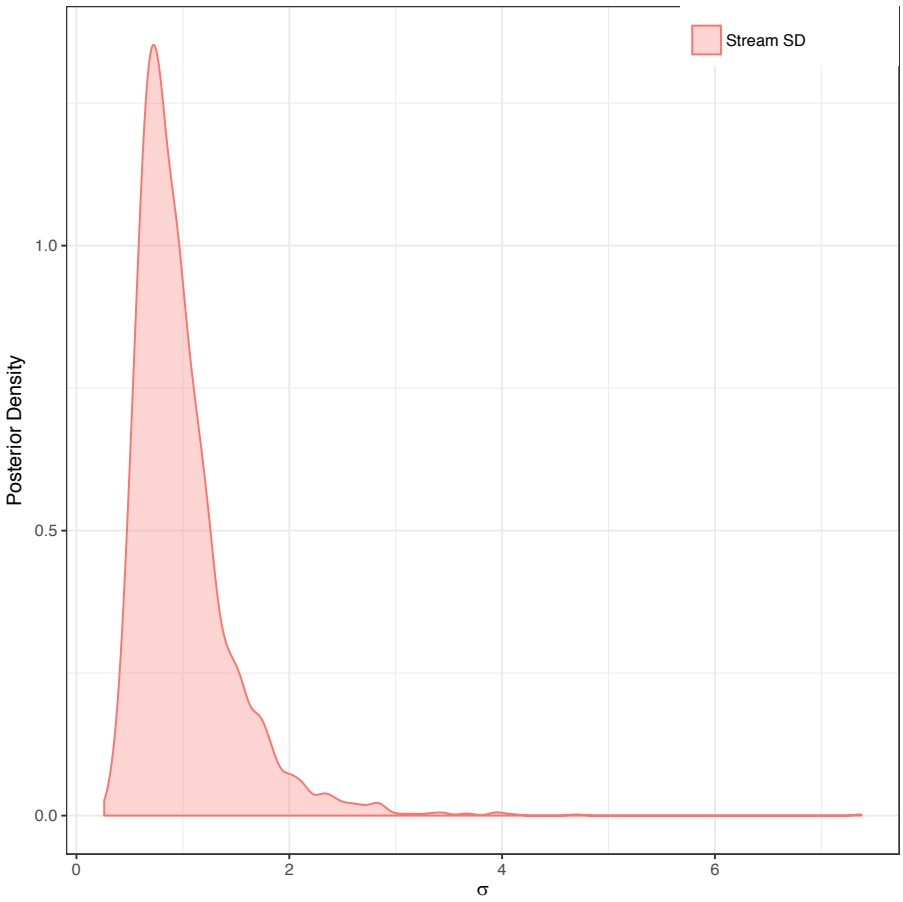


Figure S4.11 Posterior density plot of standard deviations for $\delta^{15}N$ and $\delta^{13}C$ model


Stream SD
Posterior Density
1.0
0.5
0.0
0
2
4
6
σ

Supplementary Tables

Table S4.1 Estimates of arthropod percent lipid (per dry biomass), percent ALA, and percent EPA used in calculating insect fluxes. A is aquatic and T is terrestrial.

Taxa	Lipid (%)	Source	ALA (%)	EPA (%)	Source
Ephemeroptera	14.5	Hanson et al. 1985	6.919	22.325	This study
Trichoptera	18	Hanson et al. 1985	9.060	12.043	Twining, unpubl.
Plecoptera	11.903	Mean lipid value	5.305	20.531	This study
Megaloptera	7.28	Lease and Wolf 2011	6.397	21.745	Mean A values
A Diptera	9.82	Lease and Wolf 2011	6.397	21.745	Mean A values
A Coleoptera	14.01	Lease and Wolf 2011	6.397	21.745	Mean A values
A Other	11.903	Mean lipid value	6.397	21.745	Mean A values
Hymenoptera	6.44	Lease and Wolf 2011	18.325	0	This study
Lepidoptera	12.57	Lease and Wolf 2011	27.116	2.185	This study
Hemiptera	13.93	Lease and Wolf 2011	13.011	2.293	Mean T values
Orthoptera	17.76	Lease and Wolf 2011	13.011	2.293	Mean T values
Araneae	9.27	Lease and Wolf 2011	13.011	2.293	Mean T values
T Diptera	9.82	Lease and Wolf 2011	2.371	2.442	This study
T Coleoptera	14.01	Lease and Wolf 2011	2.476	4.766	This study
T Other	11.903	Mean lipid value	13.011	2.293	Mean T values

Table S4.2 General linear models of percent ALA, percent EPA, and C:N by site. SE is standard error, LSM is least squares means, and df is degrees of freedom.

ALA (percent of fatty acid composition)

	SE	LSM	t-value	p-value
Intercept	2.896	---	4.189	< 0.0001
Candor	---	12.131	---	---
Carter	3.921	9.234	-0.739	0.462
Cascadilla	4.250	9.120	-0.708	0.480
Chaffee	4.096	11.678	-0.110	0.912
Locke	3.921	13.028	0.229	0.819
Michigan	4.096	12.039	-0.022	0.982
Miller	4.031	11.715	-0.103	0.918
Wilseyville	4.250	10.077	-0.483	0.630
Null deviance: 14701 on 122 df		Residual deviance: 14467 on 115 df		

EPA (percent of fatty acid composition)

	SE	LSM	t-value	p-value
Intercept	2.430	---	3.031	< 0.005
Candor	---	7.367	---	---
Carter	3.291	9.143674	0.540	0.590
Cascadilla	3.567	5.338541	-0.569	0.571
Chaffee	3.437	4.670220	-0.785	0.434
Locke	3.291	9.792986	0.737	0.462
Michigan	3.437	7.558367	0.056	0.956
Miller	3.383	8.727109	0.402	0.688
Wilseyville	3.498	7.275967	-0.026	0.979
Null deviance: 10625 on 123 df		Residual deviance: 10278 on 116 df		

C:N

	SE	LSM	t-value	p-value
Intercept	0.137	---	34.001	< 0.0001
Candor	0.189	4.965	1.671	0.0957
Carter	0.188	4.612	-0.198	0.843
Cascadilla	0.201	4.826	0.875	0.382
Chaffee	0.200	4.889	1.199	0.231
Locke	0.190	4.799	0.787	0.432
Michigan	0.195	4.612	-0.192	0.848
Miller	---	4.649	---	---
Wilseyville	0.192	4.711	0.321	0.748
Null deviance: 251.29 on 328 df		Residual deviance: 246.10 on 321 df		

Table S4.3 General linear models of within-taxa freshwater insect and terrestrial insect percent ALA and percent EPA by site. SE is standard error, LSM is least squares means, and df is degrees of freedom. Note that no EPA was detected in bees at any site.

Terrestrial Beetles ALA

	SE	LSM	t-value	p-value	Direction
Intercept	1.0398	---	0.671	0.515	---
Candor	---	0.697	---	---	Candor,
Carter	1.471	0.824	0.086	0.933	Carter,
Cascadilla	2.079	2.696	0.961	0.355	Cascadilla,
Chaffee	1.471	1.194	0.338	0.741	Chaffee,
Locke	1.471	2.544	1.256	0.233	Michigan,
Michigan	1.471	2.975	1.549	0.147	Wilseyville
Miller	1.644	9.383	5.283	< 0.001	< Miller
Wilseyville	1.644	1.670	0.592	0.565	
Null deviance: 159.05 on 19 df			Residual deviance: 38.92 on 12 df		

Terrestrial Beetles EPA

	SE	LSM	t-value	p-value	Direction
Intercept	1.126	---	7.585	< 0.0001	---
Candor	---	8.540	---	---	Cascadilla,
Carter	1.592	8.060	-0.302	0.7681	Chaffee,
Cascadilla	2.252	1.895	-2.951	< 0.05	Locke,
Chaffee	1.592	3.564	-3.125	< 0.01	Michigan,
Locke	1.592	2.466	-3.815	< 0.01	Miller <
Michigan	1.592	4.0257	-2.835	< 0.05	Candor
Miller	1.780	2.280	-3.517	< 0.01	
Wilseyville	1.780	4.450	-2.298	< 0.05	Chaffee, Locke, Miller < Carter
Null deviance: 163.558 on 19 df			Residual deviance: 45.631 on 12 df		

Stoneflies ALA

	SE	LSM	t-value	p-value	Direction
Intercept	0.7672	---	8.956	< 0.0001	---
Carter	---		---	---	Locke <
Locke	1.085		-3.701	< 0.01	Miller,
Michigan	1.213		-0.816	0.441	Michigan,
Miller	1.085		-0.981	0.359	Carter
Null deviance: 39.133 on 10 df			Residual deviance: 12.359 on 7 df		

Stoneflies EPA

	SE	LSM	t-value	p-value	Direction
Intercept	0.9431	---	18.957	< 0.0001	---
Carter	---	17.878	---	---	Carter,
Locke	1.334	26.192	6.234	< 0.001	Michigan,
Michigan	1.491	17.658	-0.148	0.887	Miller <
Miller	1.334	19.439	1.170	0.280	Locke

Null deviance: 156.033 on 10 df			Residual deviance: 18.678 on 7 df		

Mayflies ALA

	SE	LSM	t-value	p-value	Direction
Intercept	1.478	---	4.964	< 0.001	---
Candor	---	7.335	---	---	Cascadilla,
Carter	2.090	8.951	0.773	0.451	Locke < Carter
Cascadilla	2.090	2.863	-2.140	< 0.05	
Chaffee	2.090	13.043	2.732	< 0.05	Locke, Miller,
Locke	2.090	2.451	-2.337	< 0.05	Cascadilla,
Michigan	2.336	9.193	0.795	0.439	Wilseyville
Miller	2.090	5.503	-0.877	0.394	< Chaffee
Wilseyville	2.090	6.769	-0.271	0.790	
					Locke < Michigan
Null deviance: 349.351 on 22 df			Residual deviance: 98.248 on 15 df		

Mayflies EPA

	SE	LSM	t-value	p-value	Direction
Intercept	2.0411	---	12.832	< 0.0001	---
Candor	---	26.193	---	---	Chaffee <
Carter	2.887	21.898	-1.488	0.157	Candor,
Cascadilla	2.887	18.779	-2.568	< 0.05	Locke,
Chaffee	2.887	14.993	-3.880	< 0.01	Michigan,
Locke	2.887	26.790	0.207	0.839	Wilseyville
Michigan	3.227	25.549	-0.199	0.845	
Miller	2.887	21.054	-1.780	0.0953	
Wilseyville	2.887	24.418	-0.615	0.548	
Null deviance: 530.49 on 22 df			Residual deviance: 187.48 on 15 df		

Terrestrial Flies ALA

	SE	LSM	t-value	p-value	Direction
Intercept	0.850	---	2.182	< 0.05	---
Candor	---	1.855	---	---	No significant
Carter	1.202	2.677	0.684	0.505	differences
Cascadilla	1.202	1.806	-0.040	0.968	
Chaffee	1.202	2.526	0.558	0.585	
Locke	1.202	2.427	0.476	0.641	
Michigan	1.202	4.376	2.097	0.0534	
Miller	1.202	0.809	-0.870	0.398	
Wilseyville	1.344	2.548	0.516	0.614	
Null deviance: 54.080 on 22 df			Residual deviance: 32.522 on 15 df		

Terrestrial Flies EPA

	SE	LSM	t-value	p-value	Direction
Intercept	1.49374	---	0.581	0.569	---
Candor	---	0.8681945	---	---	No significant
Carter	2.11246	2.6156517	0.827	0.420	differences
Cascadilla	2.11246	2.8565082	0.941	0.361	

Chaffee	2.11246	1.6046144	0.349	0.732	
Locke	2.11246	0.7987180	-0.033	0.974	
Michigan	2.11246	3.3450389	1.172	0.258	
Miller	2.11246	3.3682767	1.183	0.254	
Wilseyville	2.11246	4.0759899	1.519	0.148	
Null deviance: 138.37 on 23 df			Residual deviance: 107.10 on 16 df		

Bees ALA

	SE	LSM	t-value	p-value	Direction
Intercept	3.298	---	5.185	< 0.001	---
Candor	---	17.100	---	---	Candor,
Carter	4.664	18.167	0.229	0.822	Carter,
Cascadilla	4.664	13.501	-0.772	0.453	Cascadilla,
Chaffee	4.664	17.745	0.138	0.892	Chaffee,
Locke	4.664	31.601	3.109	< 0.01	Michigan,
Michigan	5.215	16.329	-0.148	0.885	Miller,
Miller	5.215	16.609	-0.094	0.926	Wilseyville
Wilseyville	4.664	14.310	-0.598	0.559	< Locke
Null deviance: 1123.2 on 21 df			Residual deviance: 456.8 on 14 df		

Terrestrial Butterflies ALA

	SE	LSM	t-value	p-value	Direction
Intercept	3.307	---	10.180	< 0.0001	---
Candor	---	33.667	---	---	Carter,
Carter	4.677	17.917	-3.367	< 0.01	Cascadilla,
Cascadilla	4.677	20.451	-2.826	< 0.05	Wilseyville
Chaffee	4.677	23.884	-2.092	0.0528	< Candor
Locke	4.677	36.289	0.561	0.583	
Michigan	4.677	31.910	-0.376	0.712	Carter,
Miller	4.677	33.035	-0.135	0.894	Cascadilla,
Wilseyville	4.677	19.778	-2.970	< 0.01	Michigan, Miller, Wilseyville < Locke Wilseyville < Miller
Null deviance: 1660.30 on 23 df			Residual deviance: 525.03 on 16 df		

Terrestrial Butterflies EPA

	SE	LSM	t-value	p-value	Direction
Intercept	0.362	---	3.407	< 0.01	---
Candor	---	1.233	---	---	Candor,
Carter	0.512	4.410	6.206	< 0.001	Cascadilla,
Cascadilla	0.512	0.866	-0.718	0.483	Locke,
Chaffee	0.512	3.189	3.821	< 0.01	Michigan,
Locke	0.512	2.511	2.495	< 0.05	Miller,
Michigan	0.512	1.616	0.748	0.465	Wilseyville
Miller	0.512	1.163	-0.137	0.893	< Carter

Wilseyville	0.512	2.494	2.462	< 0.05	Cascadilla < Chaffee, Locke, Wilseyville Michigan, Miller < Chaffee
Null deviance: 36.815 on 23 df			Residual deviance: 6.290 on 16 df		

Supplementary Table 4.4 Mean (and one standard error) of omega-3 and omega-6 fatty acids in Eastern Phoebe chick blood. All data is expressed as percent of total fatty acid composition. Total n-3 is total omega-3 fatty acids and total n-6 is total omega-6 fatty acids. Blood samples contained only trace amounts of EPA.

	Miller	**West Candor**	**Locke**
18:2n-6	12.64 (0.27)	13.03 (0.19)	14 (0.35)
18:3n-6	0.14 (0.02)	0.16 (0.03)	0.19 (0.05)
ALA (18:3n-3)	10.09 (0.84)	9.31 (0.81)	14.26 (0.62)
20:2n-6	0.41 (0.11)	0.46 (0.04)	0.67 (0.09)
20:3n-6	0.68 (0.06)	0.54 (0.06)	0.92 (0.09)
20:4n-6	8.92 (0.26)	3.60 (0.33)	7.46 (0.35)
20:3n-3	0.18 (0.04)	0.29 (0.06)	0.59 (0.07)
DPA (22:5n-3)	1.61 (0.18)	1.26 (0.10)	2.19 (0.05)
DHA (22:6n-3)	4.52 (0.11)	3.20 (0.14)	4.00 (0.12)
Total n-3	16.40 (0.75)	14.06 (0.72)	14.26 (0.62)
Total n-6	22.79 (0.59)	17.80 (0.41)	23.23 (0.15)

Table S4.5 Linear mixed effects models of freshwater insect biomass fluxes and terrestrial arthropod biomass fluxes by date, by site, and by taxa. DOY is day of year, B is the fixed effect vector estimate, σ^2 is within-group variance, τ is between-group variance, N is number of factor levels (i.e., number of streams and number of stream by taxon combinations), and ICC is intra-class correlation.

Freshwater Biomass Fluxes

Fixed Effect	B	p-value	Fixed Effect	B	p-value
Intercept	0.48	< 0.05	Coleoptera	-0.28	0.79
DOY 145	0.03	0.88	Ephemeroptera	0.24	0.38
DOY 150	0.29	< 0.05	Megaloptera	-0.36	0.90
DOY 151	-0.28	0.73	Nematocera	0.27	0.98
DOY 152	-0.28	0.73	Other	-0.60	0.46
DOY 154	-0.31	0.05	Plecoptera	0.34	0.42
DOY 155	-0.12	0.40	Trichoptera	0.07	0.08
DOY 163	-0.06	0.62			
Random Effects				$R^2 = 0.25$	
σ^2	0.63				
$\tau_{, \text{Taxon:Stream}}$	0.08		$\tau_{, \text{Stream}}$	0.04	
$N_{\text{Taxon:Stream}}$	44		N_{Stream}	8	
$ICC_{\text{Taxon:Stream}}$	0.10		ICC_{Stream}	0.05	

Freshwater ALA Fluxes

Fixed Effect	B	p-value	Fixed Effect	B	p-value
Intercept	0.01	0.58	Coleoptera	-0.01	0.79
DOY 145	0.00	0.92	Ephemeroptera	0.01	0.38
DOY 150	0.03	< 0.001	Megaloptera	0.00	0.90
DOY 151	0.00	0.99	Nematocera	0.00	0.98
DOY 152	0.00	0.99	Other	-0.02	0.46
DOY 154	0.00	0.70	Plecoptera	0.01	0.42
DOY 155	0.00	0.70	Trichoptera	0.03	0.08
DOY 163	0.00	0.56			
Random Effects				$R^2 = 0.25$	
σ^2	0.003				
$\tau_{, \text{Taxon:Stream}}$	0.00		$\tau_{, \text{Stream}}$	0.00	
$N_{\text{Taxon:Stream}}$	44		N_{Stream}	8	
$ICC_{\text{Taxon:Stream}}$	0.13		ICC_{Stream}	0.01	

Freshwater EPA Fluxes

Fixed Effect	B	p-value	Fixed Effect	B	p-value
Intercept	0.02	0.28	Coleoptera	-0.02	0.70
DOY 145	0.00	0.95	Ephemeroptera	0.04	0.17
DOY 150	0.05	< 0.01	Megaloptera	-0.01	0.82
DOY 151	-0.01	0.93	Nematocera	0.00	0.87
DOY 152	-0.01	0.93	Other	-0.04	0.28
DOY 154	-0.01	0.62	Plecoptera	0.04	0.09
DOY 155	0.00	0.88	Trichoptera	0.01	0.59
DOY 163	-0.01	0.54			
Random Effects				$R^2 = 0.19$	
σ^2	0.01				
$\tau_{, \text{Taxon:Stream}}$	0.001		$\tau_{, \text{Stream}}$	0.00	

$N_{Taxon:Stream}$	44	N_{Stream}	8
$ICC_{Taxon:Stream}$	0.09	ICC_{Stream}	0.02

Terrestrial Biomass Fluxes

Fixed Effect	B	p-value	Fixed Effect	B	p-value
Intercept	0.42	< 0.05	Hemiptera	-0.63	< 0.01
DOY 145	0.06	0.76	Hymenoptera	-0.17	0.39
DOY 150	1.01	< 0.001	Lepidoptera	-0.25	0.21
DOY 154	0.23	0.46	Orthoptera	-0.65	< 0.05
DOY 163	-0.06	0.64	Other	-0.54	< 0.05
Coleoptera	1.47	< 0.001	Thysanoptera	-0.63	< 0.05
Diptera	-0.14	0.47			
Formicidae	-0.55	< 0.05		$R^2 = 0.38$	
Random Effects					
σ^2	1.14				
$\tau_{, Taxon:Stream}$	0.01		$\tau_{, Stream}$	0.00	
$N_{Taxon:Stream}$	57		N_{Stream}	8	
$ICC_{Taxon:Stream}$	0.004		ICC_{Stream}	0.00	

Terrestrial ALA Fluxes

Fixed Effect	B	p-value	Fixed Effect	B	p-value
Intercept	0.05	0.09	Hemiptera	-0.09	< 0.05
DOY 145	0.00	0.92	Hymenoptera	-0.06	0.09
DOY 150	0.16	< 0.001	Lepidoptera	-0.04	0.33
DOY 154	0.02	0.72	Orthoptera	-0.10	0.07
DOY 163	0.00	0.95	Other	-0.10	< 0.05
Coleoptera	0.09	< 0.05	Thysanoptera	-0.10	0.07
Diptera	-0.10	< 0.05			
Formicidae	-0.10	< 0.05		$R^2 = 0.21$	
Random Effects					
σ^2	0.04				
$\tau_{, Taxon:Stream}$	0.00		$\tau_{, Stream}$	0.00	
$N_{Taxon:Stream}$	57		N_{Stream}	8	
$ICC_{Taxon:Stream}$	0.01		ICC_{Stream}	0.00	

Terrestrial EPA Fluxes

Fixed Effect	B	p-value	Fixed Effect	B	p-value
Intercept	0.00	0.66	Hemiptera	0.00	0.83
DOY 145	0.00	0.88	Hymenoptera	0.00	0.70
DOY 150	0.00	< 0.001	Lepidoptera	0.00	0.88
DOY 154	0.00	0.79	Orthoptera	0.00	0.82
DOY 163	0.00	0.89	Other	0.00	0.75
Coleoptera	0.00	< 0.001	Thysanoptera	0.00	0.81
Diptera	0.00	0.73			
Formicidae	0.00	0.76		$R^2 = 0.23$	
Random Effects					
σ^2	0.00				
$\tau_{, Taxon:Stream}$	0.00		$\tau_{, Stream}$	0.00	
$N_{Taxon:Stream}$	57		N_{Stream}	8	
$ICC_{Taxon:Stream}$	0.05		ICC_{Stream}	0.00	

Table S4.6 Fraction of Eastern Phoebe chick diet from freshwater or terrestrial prey based on $\delta^{15}N$ and $\delta^{13}C$ and on $\delta^{15}N$ and $\delta^{2}H$ mixing model results and diagnostics

	$\delta^{15}N$ and $\delta^{13}C$ Model Estimate	**$\delta^{15}N$ and $\delta^{13}C$ Estimate SD**	**$\delta^{15}N$ and $\delta^{2}H$ Model Estimate**	**$\delta^{15}N$ and $\delta^{2}H$ Estimate SD**
Overall freshwater prey	0.477	0.115	0.457	0.090
Overall terrestrial prey	0.523	0.115	0.543	0.090
Miller freshwater prey	0.289	0.057	0.329	0.065
Michigan freshwater prey	0.253	0.118	0.430	0.130
Wilseyville freshwater prey	0.394	0.089	0.344	0.067
Candor freshwater prey	0.718	0.018	0.703	0.017
Chaffee freshwater prey	0.357	0.066	0.372	0.063
Carter freshwater prey	0.866	0.066	0.766	0.086
Cascadilla freshwater prey	0.388	0.111	0.377	0.106
Locke freshwater prey	0.421	0.041	0.360	0.035
Miller terrestrial prey	0.711	0.057	0.671	0.065
Michigan terrestrial prey	0.747	0.118	0.570	0.130
Wilseyville terrestrial prey	0.606	0.089	0.656	0.067
Candor terrestrial prey	0.282	0.018	0.297	0.017
Chaffee terrestrial prey	0.643	0.066	0.628	0.063
Carter terrestrial prey	0.134	0.066	0.234	0.086
Cascadilla terrestrial prey	0.612	0.111	0.623	0.106
Locke terrestrial prey	0.579	0.041	0.640	0.035
	Diagnostics for $\delta^{15}N$ and $\delta^{13}C$ Model			
Gelman-Rubin	Out of 22 variables: 3 > 1.01, 0 > 1.05, 0 > 1.1			
Geweke	Number of variables outside +/-1.96 in each chain: Chain 1 = 4, Chain 2 = 5, Chain 3 = 2			

Table S4.7 General linear models of percent of Eastern Phoebe chick diet from freshwater prey versus all possible explanatory variables. SE is standard error, NS is not significant, and df is degrees of freedom.

FORAGING BEHAVIOR

Stream Foraging

	SE	t-value	p-value	Direction
Intercept	7.388	6.042	< 0.0001	---
Stream Foraging	0.244	1.202	0.241	NS
Null deviance: 14836 on 26 df		Residual deviance: 14025 on 25 df		

Riparian Foraging

	SE	t-value	p-value	Direction
Intercept	9.159	8.397	< 0.0001	---
Riparian Foraging	0.176	-3.067	< 0.01	positive
Null deviance: 14836 on 26 df		Residual deviance: 10780 on 25 df		

Terrestrial Foraging

	SE	t-value	p-value	Direction
Intercept	6.511	6.947	< 0.0001	---
Terrestrial Foraging	0.159	1.366	0.184	NS
Null deviance: 14836 on 26 df		Residual deviance: 13806 on 25 df		

Stream and Riparian Foraging

	SE	t-value	p-value	Direction
Intercept	12.097	5.535	< 0.0001	---
Str. + Rip. Foraging	0.159	-1.366	0.184	NS
Null deviance: 14836 on 26 df		Residual deviance: 13806 on 25 df		

PREY AVAILABILITY

Mean Freshwater Biomass

	SE	t-value	p-value	Direction
Intercept	7.0483	10.240	< 0.0001	---
Mean Fresh. Biomass	0.4548	-4.031	< 0.001	negative
Null deviance: 18095 on 34 df		Residual deviance: 12125 on 33 df		

Maximum Freshwater Biomass

	SE	t-value	p-value	Direction
Intercept	6.561	9.328	< 0.0001	---
Max. Fresh. Biomass	0.0934	-2.602	< 0.05	negative
Null deviance: 18095 on 34 df		Residual deviance: 15015 on 33 df		

Mean Terrestrial Biomass

	SE	t-value	p-value	Direction
Intercept	6.217	8.222	< 0.0001	---
Mean Terr. Biomass	0.0398	-0.865	0.393	NS
Null deviance: 18095 on 34 df		Residual deviance: 17693 on 33 df		

Maximum Terrestrial Biomass

	SE	t-value	p-value	Direction
Intercept	6.650	7.763	< 0.0001	---
Max. Terr. Biomass	0.00892	-0.871	0.390	NS
Null deviance: 18095 on 34 df		Residual deviance: 17689 on 33 df		

PREY QUALITY AND AVAILABILITY

Mean Freshwater ALA Flux

	SE	t-value	p-value	Direction
Intercept	6.804	9.896	< 0.0001	---
Fresh. ALA Flux	48.649	-3.457	< 0.01	negative
Null deviance: 18095 on 34 df		Residual deviance: 13284 on 33 df		

Mean Freshwater EPA Flux

	SE	t-value	p-value	Direction
Intercept	6.918	10.270	< 0.0001	---
Fresh. EPA Flux	17.392	-3.951	< 0.001	negative
Null deviance: 18095 on 34 df		Residual deviance: 12284 on 33 df		

Mean Terrestrial ALA Flux

	SE	t-value	p-value	Direction
Intercept	6.322	9.125	< 0.0001	---
Terr. ALA Flux	7.734	-2.102	< 0.05	negative
Null deviance: 18095 on 34 df		Residual deviance: 15959 on 33 df		

Mean Terrestrial EPA Flux

	SE	t-value	p-value	Direction
Intercept	5.860	8.419	< 0.0001	---
Terr. EPA Flux	6113.49	-0.553	0.584	NS
Null deviance: 18095 on 34 df		Residual deviance: 17929 on 33 df		

PREY QUALITY

Mean Freshwater Percent ALA

	SE	t-value	p-value	Direction
Intercept	10.055	4.105	< 0.001	---
Fresh. Percent ALA	1.300	0.612	0.545	NS
Null deviance: 18095 on 34 df		Residual deviance: 17892 on 33 df		

Mean Freshwater Percent EPA

	SE	t-value	p-value	Direction
Intercept	22.755	1.280	0.210	---
Fresh. Percent EPA	1.034	0.795	0.432	NS
Null deviance: 18095 on 34 df		Residual deviance: 17755 on 33 df		

Mean Terrestrial Percent ALA

	SE	t-value	p-value	Direction
Intercept	19.380	4.477	< 0.0001	---
Terr. Percent ALA	1.484	-2.094	< 0.05	negative
Null deviance: 18095 on 34 df		Residual deviance: 5973 on 33 df		

Mean Terrestrial Percent EPA

	SE	t-value	p-value	Direction
Intercept	9.701	-0.429	0.671	---

Terr. Percent EPA	3.909	5.512	< 0.0001	positive
Null deviance: 18095 on 34 df		Residual deviance: 9421.4 on 33 df		

LAND USE AND LIGHT AVAILABILITY

Agricultural Land Use within 100 m radius

	SE	t-value	p-value	Direction
Intercept	5.766	9.550	< 0.0001	---
Agriculture	0.152	-1.860	0.0718	NS
Null deviance: 18095 on 34 df		Residual deviance: 16377 on 33 df		

Agricultural Land Use within 25 m buffer zone

	SE	t-value	p-value	Direction
Intercept	4.469	13.171	< 0.0001	---
Agriculture	0.204	-3.915	< 0.001	negative
Null deviance: 18095 on 34 df		Residual deviance: 12356 on 33 df		

Stream Canopy Cover

	SE	t-value	p-value	Direction
Intercept	7.930	5.870	< 0.0001	---
Cover	0.133	0.0560	0.955	NS
Null deviance: 18095 on 34 df		Residual deviance: 18093 on 33 df		

TEMPERATURE AND STREAM SIZE

Stream Discharge

	SE	t-value	p-value	Direction
Intercept	6.055	5.912	< 0.0001	---
Stream Discharge	5.514	2.315	< 0.05	positive
Null deviance: 18095 on 34 df		Residual deviance: 15567 on 33 df		

Stream Temperature

	SE	t-value	p-value	Direction
Intercept	40.357	2.477	< 0.05	---
Stream Temperature	2.503	-1.320	0.196	NS
Null deviance: 18095 on 34 df		Residual deviance: 17188 on 33 df		

STREAM PRIMARY PRODUCTION AND PRODUCERS

Stream Dissolved Oxygen (DO)

	SE	t-value	p-value	Direction
Intercept	42.100	6.010	< 0.0001	---
DO	4.624	-4.907	< 0.0001	negative
Null deviance: 18095 on 34 df		Residual deviance: 10461 on 33 df		

Stream DO Coefficient of Variation

	SE	t-value	p-value	Direction
Intercept	7.899	4.497	< 0.0001	---
DO CV	69.102	1.649	0.109	NS
Null deviance: 18095 on 34 df		Residual deviance: 16717 on 33 df		

Chlorophyll a

	SE	t-value	p-value	Direction
Intercept	5.253	9.427	< 0.0001	---

Chlorophyll a	1.873	-0.739	0.465	NS
Null deviance: 18095 on 34 df		Residual deviance: 17800 on 33 df		

Ash Free Dry Mass (AFDM)

	SE	t-value	p-value	Direction
Intercept	5.645	7.699	< 0.0001	---
AFDM	3.365	0.855	0.398	NS
Null deviance: 18095 on 34 df		Residual deviance: 17702 on 33 df		

STREAM WATER CHEMISTRY

Stream Total Phosphorus

	SE	t-value	p-value	Direction
Intercept	7.454	7.726	< 0.0001	---
Total Phosphorus	0.0864	-1.661	0.106	NS
Null deviance: 18095 on 34 df		Residual deviance: 16699 on 33 df		

Stream Soluble Reactive Phosphorus (SRP)

	SE	t-value	p-value	Direction
Intercept	10.615	4.744	< 0.0001	---
SRP	0.884	-0.346	0.731	NS
Null deviance: 18095 on 34 df		Residual deviance: 18029 on 33 df		